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TITLE: Enhanced Androgen Signaling with Androgen Receptor Overexpression in the Osteoblast Lineage Controls Skeletal Turnover, Matrix Quality and Bone Architecture

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14. ABSTRACT
Androgens have been shown to be important mediators of bone growth and remodeling independent of estrogen. We genetically engineered transgenic mice in which androgen receptor (AR) overexpression is skeletally targeted in two separate models to better understand the role of androgen signaling directly in bone. In the fourth year, we have published the analysis of the second line of AR-transgenic mice, AR2.3-transgenic mice. Enhanced androgen signaling directly in bone results in inhibition of bone formation by differentiated osteoblasts, with a phenotype reflecting low turnover. Comparisons between both models of AR2.3- and AR3.6-transgenic animals suggests that AR transactivation in osteocytes is primarily responsible for mediating the effects of androgen on matrix quality and/or mineralization (inhibitory), while stromal/immature cells mediate effects of androgen on the periosteum and body composition (anabolic). The consequence of androgen action *in vivo* is compartment-specific; anabolic effects are exhibited exclusively at periosteal surfaces, but in mature osteoblasts androgens inhibit osteogenesis with detrimental effects on matrix quality, bone fragility and whole bone strength (Specific Aim 1). Gene expression profiling has identified important signaling pathways by which androgens influence osteoblast-osteoclast communication (Specific aim 2). *Ex vivo* differentiation analysis of proliferation and differentiation in calvarial cultures from AR3.6-tg and AR2.3-tg mice demonstrates inhibition of proliferation that is not affected by the AR-tg (likely because expression levels are low in proliferating cultures), and robust inhibition of osteoblast matrix maturation and mineralization that is most severe in cultures with AR overexpression throughout the osteoblast lineage (Specific aim 3).

15. SUBJECT TERMS
Androgen, Androgen receptor, Transgenic, Bone mass, Bone quality, Biomechanics

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Introduction

Androgen deficiency (as a result of aging, hypogonadism, glucocorticoid therapy, or alcoholism), and other behaviors (chronic smoking, malabsorption and bone marrow malignancies) are associated with the development of osteoporosis in men (1). Osteoporosis is also an important and debilitating side effect of androgen deprivation therapy in conjunction with the treatment of prostate cancer (2, 3). At any one time, osteoporosis affects 20 million Americans. Nearly one-quarter of the patients who suffer a hip fracture die within the first year; 50% of patients are unable to walk without assistance; and 33% are totally dependent (4, 5). Of the 1.3 million bone fractures that can be attributed to osteoporosis every year, 150,000 are hip fractures that occur in men with lifetime risk for the development of fracture at nearly 15% (6). In addition, it is also clear that androgens have an important but very much under-appreciated role in women (7). Other health problems may also be affected by androgen action, including atherosclerotic vascular disease, age-related weakness and disability, memory loss, etc. Since osteoporosis is often coupled with a hypogonadal state, developing an understanding of androgen action in the skeleton may provide insight into development of novel therapeutics for the treatment of osteoporosis and metabolic bone disease.

The distinct contribution of androgen to the maintenance of a healthy skeleton remains controversial, since the major androgen metabolite testosterone can serve as the substrate for the production estradiol via aromatase activity. As a consequence, some testosterone action may result from estrogen receptor-dependent activation after conversion to 17-ß estradiol. Overexpression of androgen receptor (AR), combined with the use of non-aromatizable androgens that cannot serve as a substrate for aromatase conversion (e.g. 5α-dihydrotestosterone; DHT), should enhance our understanding of the *specific* role for androgen in bone biology. The goal of this program is to gain a comprehensive understanding of the cascade of molecular and cellular events by which androgen signaling influences skeletal homeostasis.

Our proposed studies have substantial military significance. The stated goals of the Bone Health and Military Readiness program are to advance the understanding of methods to improve bone health of young men and women, to enhance military readiness by reducing the incidence of fracture during physically intensive training, and to reduce the incidence of osteoporosis later in life. As little is known about the direct actions of androgens on osteoblasts, our comprehensive approach using unique animal models of enhanced androgen responsiveness with distinct bone-targeted AR-transgenic families, combined with the novel studies of DHT modulation of osteoblast differentiation and osteoblast-osteoclast signaling, will provide insights into normal bone homeostasis. Understanding the consequences of androgen action in bone is particularly important given increased anabolic steroid abuse. In addition, since bone architecture and bone material properties play important roles in stress fracture, analysis of this model represents a unique opportunity to characterize the consequences of androgen action in both genders on bone microarchitectural quality and the integrity of the skeleton *in vivo*.

Body

In the last year of this grant, we have published the characterization of signaling pathways that are implicated in the inhibitory actions of androgen in cortical bone (as outlined in Specific Aim 3) and the cell-autonomous inhibitory action in primary osteoblasts of androgen treatment on proliferation and differentiated function (as outlined in Specific Aim 3). Thus, we have employed the two distinct AR-transgenic families that we constructed to better characterize the direct effects of androgen signaling in the skeleton. The two lines include the AR3.6-transgenic families with AR overexpression in stromal cells and throughout the osteoblast lineage including

mature osteoblasts, and the AR2.3-transgenic mice with overexpression limited to the mature osteoblast/osteocyte. These mice provide models for the characterization of enhanced androgen signaling in distinct skeletal compartments *in vivo*, through a comparison the phenotypes observed in both lines (see summary illustration below).

    Importantly in both models, enhanced androgen action occurs only in those cells with elevated levels of AR (skeletally-targeted) as a consequence of enhanced AR signaling, without changes in circulating steroid levels and without systemic androgen administration. Because of distinct and overlapping expression profiles as shown in the schematic below, comparison of the skeletal phenotypes characterized in these two models of enhanced androgen action are postulated to aid in the identification of cells within the osteoblast lineage that are most important for mediating a specific response in bone modeling/remodeling characteristics. For example, phenotypes identified as similar in both AR2.3- transgenic and AR3.6-transgenic suggests that mature osteoblasts/osteocytes are important mediators of the response, since there is overlap in promoter activity in those cell types. In contrast, phenotypes that are more pronounced in AR3.6- transgenic compared to AR2.3-transgenic suggest that stromal or immature osteoblasts are primary mediators.
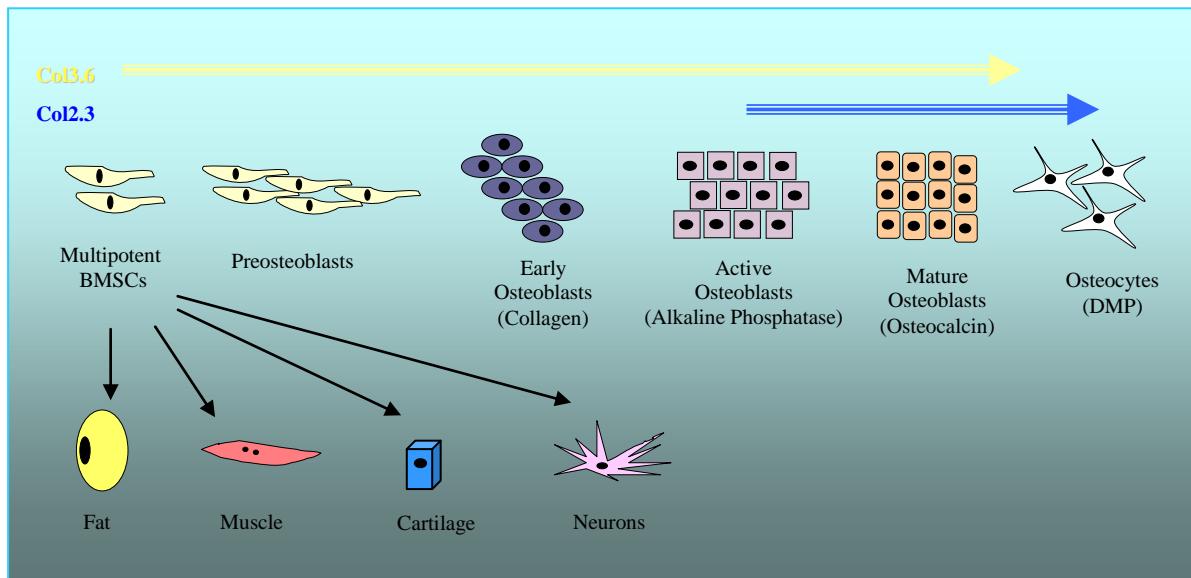
    Our goal as proposed in Specific Aim 1 was to contrast the skeletal phenotype of AR2.3-transgenic with AR3.6-transgenic animals (with different AR overexpression profiles in the osteoblast lineage), in the adult and in the hypogonadal state in both genders, to identify direct androgen actions *in vivo*. This analysis allowed us to test the hypothesis that distinct profiles of AR overexpression in the osteoblast lineage will result in distinct skeletal phenotypes between AR2.3-transgenic vs. AR3.6-transgenic mice. The final progress report is divided into three sections, with a focus on new findings:

Specific aim 1). Studies proposed in Specific aim 1 were to develop an understanding of the direct effects of androgen on the skeleton *in vivo*, through a process that compared and contrasted the phenotype in AR2.3-transgenic and AR3.6-transgenic lines (manuscripts published; Appendix 1, 2, 5, 6, 7). New analysis presented in this final progress report include: a) completion of analysis of the adult AR2.3-transgenic mouse model and comparison with AR3.6-transgenic mice (manuscripts published; Appendix 2); a) analysis of DHT replacement

studies in both a low and high bone turnover situation after gonadectomy in both males and females in both AR2.3- and AR3.6-transgenic mice for evaluation of bone parameters (complete for DXA; significant progress for µCT); b) new direction, analysis of body composition changes in low and high turnover gonadectomized mice (complete); c) new direction, analysis of the consequences of androgen receptor overexpression in brain on liability for damage after ischemic stroke.

Specific aim 2). Studies proposed in Specific aim 2 were to determine the importance of AR in regulating osteoclast formation and activation, with a focus on osteoblast-osteoclast communication (manuscripts published; Appendix 1, 2, 6, 7). New studies include: a) gene expression profiling employing quantitative RT-PCR array (qPCR) analysis to identify important signal transduction pathways altered in osteoblasts/osteocytes that mediate the inhibitory effects of androgen on osteoblast vigor and on osteoclastogenesis and/or osteoclast activity in qPCR array. These studies employed an *in vivo* analysis using whole cortical bone from which the periosteum was stripped, marrow elements flushed and metaphyses removed. This allowed a focus on osteoblast/osteocyte expression in an *in vivo* system to identify expression differences mediating osteoblast-osteoclast communication.

Specific aim 3). Studies proposed in Specific aim 3 to characterize the role of androgen in the regulation of osteoblast differentiation (manuscripts published; Appendix 3, 4, 7). New studies include: a) completion of characterization of the effects of DHT treatment on proliferation, osteoblast differentiation and mineralization in AR3.6- and AR2.3-transgenic calvarial mouse osteoblast (mOB) primary cultures; b) characterization of androgen anabolic action by array analysis in periosteal bone to identify pathways important in osteoblastogenesis; c) new direction, characterization of effects of androgen in neural crest stem cells vs. mesenchymal stem cells (MSC) *in vivo* to contribute to androgen anabolic action to enhance osteogenesis; and d) characterization of effects of androgen on MSC colony forming units and lineage commitment *in vitro*. In this final report, we have included data figures only for results that are currently unpublished.

SECTION 1: STUDIES PROPOSED IN SPECIFIC AIM 1
Analyze the consequences of enhanced androgen signaling in bone employing AR2.3-transgenic and AR3.6-transgenic models

New Studies:

1a) *Analysis of bone parameters with DHT replacement in adult gonadecomized AR-transgenic mice (both AR2.3-tg and AR3.6-tg lines) in the low and high turnover paradigms:*

A second important goal of our studies was the characterization of androgen signaling in the adult, contrasted with the effects of enhanced signaling we have characterized during skeletal development in the AR2.3- and AR3.6-transgenic mice. With the loss of gonadal steroids (during menopause, andropause or surgical castration), bone turnover is immediately increased dramatically (including increased bone resorption) such that bone strength is reduced. This high turnover state does not persist indefinitely however, and after ~1 year in humans (or ~2 months in mice), turnover rates return to near baseline. We focused on two aspects of the phenotype we have observed in developing AR2.3- and AR3.6-transgenic mice: inhibition of bone turnover (both formation and resorption) at endosteal surfaces (and in trabecular bone), with mild stimulation of bone formation at periosteal surfaces and in calvaria. The hypothesis tested is that DHT transactivation of AR will protect against hypogonadal bone loss following

gonadecomy [either ovariectomy (OVX) or orchidectomy (ORX)] in both female and male mice through inhibition of osteoclastogenesis and/or osteoclast activity in wild-type mice. However, we propose that androgen will not display anabolic activity in terms of the osteoblast but will promote bone formation via the periosteal fibroblast. Thus, increases in bone formation rate are observed exclusively on periosteal surfaces on cortical bone and in calvaria.

Two experimental approaches were taken to separately test for anti-resorptive and anabolic actions of androgen replacement in the adult, in both AR2.3- and AR3.6-transgenic male and female mice. **In the first paradigm, protracted hormone ablation to allow development of a hypogonadal phenotype was followed by steroid replacement**. Both male and female wild-type control (B6D2F2) and AR-transgenic mice were sham operated or gonadectomized at 3 months of age, and the effect of nonaromatizable dihydrotestosterone (DHT) or placebo was determined after an 8-week delay, allowing for gonadectomy-induced changes to develop. In this setting, hormone administration can be considered as a *therapeutic* measure. In the **second paradigm, hormone ablation as a consequence of gonadecomy at 5 months of age was followed immediately by steroid replacement**, again for 6 weeks. For both models following 6 weeks of treatment, micro-Computed Tomography (µCT) analysis was used to evaluate the effects of androgen on bone characteristics, and by dual energy x-ray absorptiometry (DXA) to evaluate changes in bone mineral and whole body composition.   The second approach is characterized as *preventative*. Importantly, both groups of animals are analyzed at 6.5 months of age.

Therapeutic approach to reverse hypogonadal bone loss (in low turnover/anabolic model): In this section, we present both µCT and DXA analysis for potential prevention of hypogonadal bone loss in adults. After presentation of the analysis of bone metabolism, in the next section we also present data for changes in body composition mediated by androgen signaling.

Anabolic (increased bone formation) effects were examined in male and female mice castrated at ~3 months (initially in wild-type littermate controls) with steroid pellet replacement delayed for 2 months to allow turnover to stabilize. DHT was delivered for ~6 weeks, and mice were then evaluated for changes in bone mineral by DXA using a mouse PIXImus2 densitometer to determine total body BMD and BMC and bone area. As our planned histomorphometric analysis was not completed in a timely fashion, the subcontract for that work was awarded last year to a different laboratory (at Oregon State University). Mouse bones for the high-turnover and low-turnover studies have been transferred to OSU to Drs. Iwaniec and Turner, and µCT analyses are now being completed. The µCT analysis employs a Scanco µCT40 scanner to determine cortical thickness of the femoral midshaft (20-µm voxel size) and cancellous bone volume (16-µm voxel size) in the distal femoral metaphysis and fifth LV. A threshold of 240 was used for evaluation of all scans. Twenty slices (0.4 mm) were analyzed in the midshaft femur. For the femoral metaphysis, 100 slices (1.66 mm) were analyzed, starting with the first slice in which both condyles were no longer visible. The small amount of primary spongiosa present in the first few slices was not analyzed. Analyses of LV 5 included the entire region of secondary spongiosa between proximal and distal slices in which the secondary spongiosa occupied at least 50% of the cancellous bone area. For LV 5, typically 120 slices (~2 mm) were analyzed.

First, we present the final data for DXA analysis for LOW TURNOVER bone mineral determinations in the following figures (Figs. 1-3 for males; Figs. 4-6 for females). We then present the complete analysis of bone morphology by µCT analysis for wild-type and AR2.3-tg mice (Figs. X-X).

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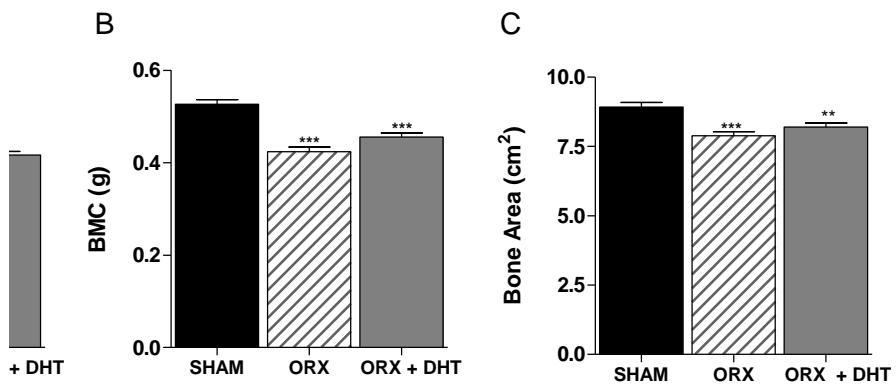
Fig. 1. Wild-type male low turnover bone mineral measures assessed by DXA. A, Whole body BMD, B, BMC and C, bone area * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Sham controls (n = 28-33)
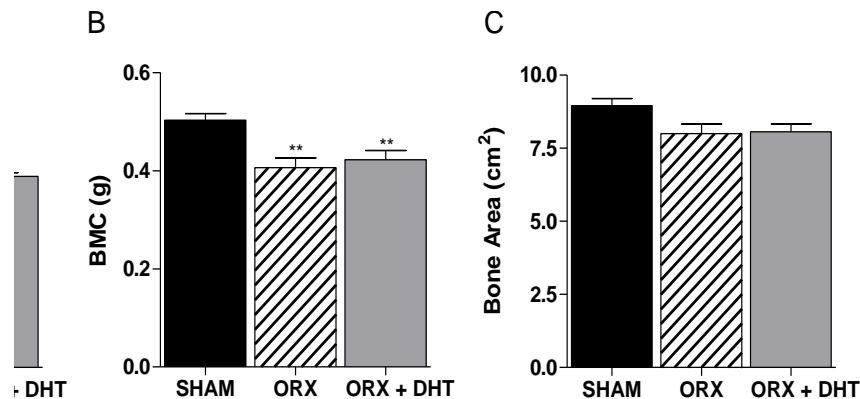

Fig. 2. AR3.6-tg male low turnover bone mineral measures assessed by DXA. A, Whole body BMD, B, BMC and C, bone area. One-way ANOVA revealed significant differences in BMD ($p < 0.01$), BMC ($p < 0.001$), and bone area ($p < 0.05$) following ORX. Tukey's multiple comparison test ** $p < 0.01$, vs. Sham controls (n = 10-11).
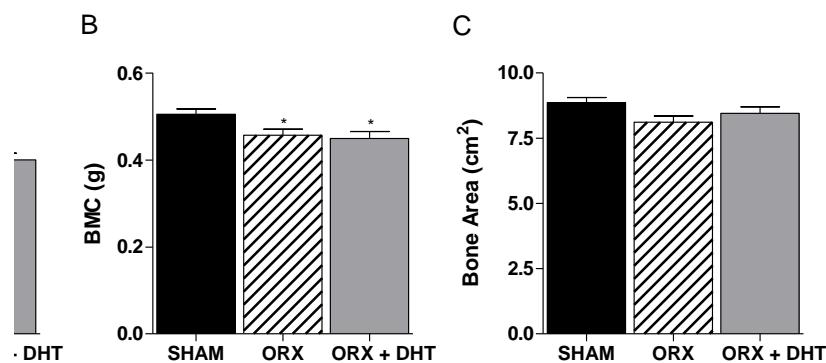

Fig. 3. AR2.3-tg male low turnover bone mineral measures assessed by DXA. A, Whole body BMD, B, BMC and C, bone area. * $p < 0.05$ (n = 10-12).

FEMALES
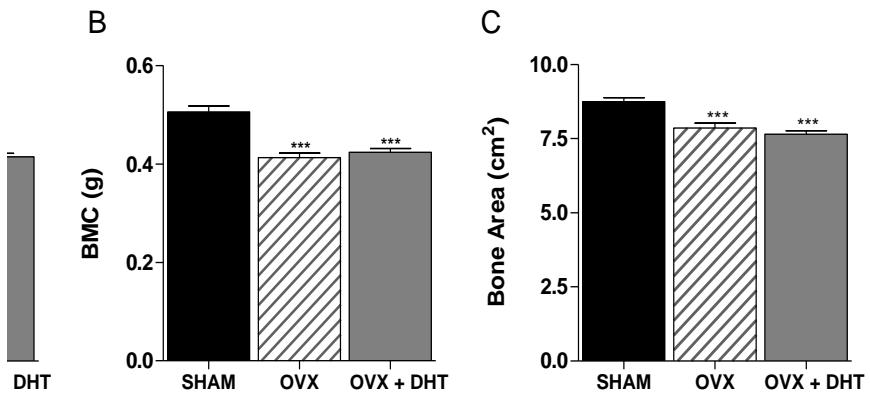
Fig. 4. Wild-type female low turnover bone mineral measures assessed by DXA. A, Whole body BMD, B, BMC and C, bone area. ** p < 0.01, *** p < 0.001 vs. Sham controls (n = 29-38).
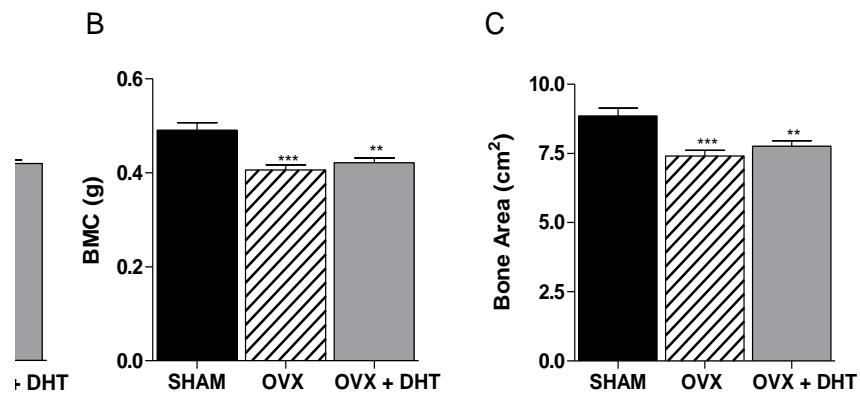

Fig. 5. AR3.6-tg female low turnover bone mineral measures assessed by DXA. A, Whole body BMD, B, BMC and C, bone area. One way ANOVA revealed significantly reduced BMC and bone area (p < 0.001) following OVX. Tukey's multiple comparison test ** p < 0.01, *** p < 0.001, vs. Sham controls (n = 12-15).
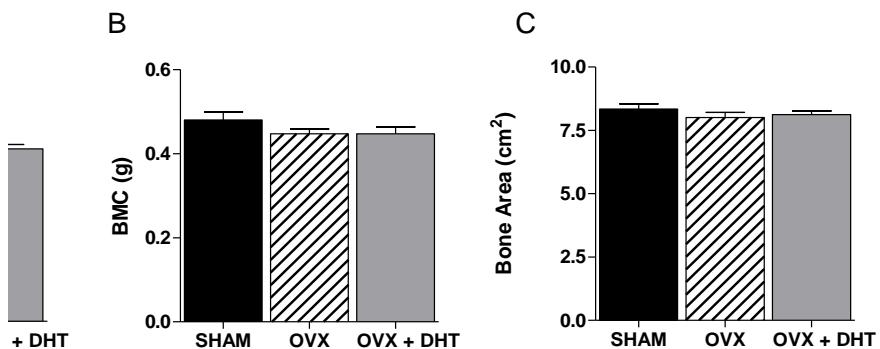

Fig. 6. AR2.3-tg female low turnover bone mineral measures assessed by DXA. A, Whole body BMD, B, BMC and C, bone area (n = 8-13).

Analysis of bone morphology with µCT analysis is now completed for *low* turnover male and female wild-type and AR2.3-tg. This data is shown in the following graphs (Fig. 7-18).

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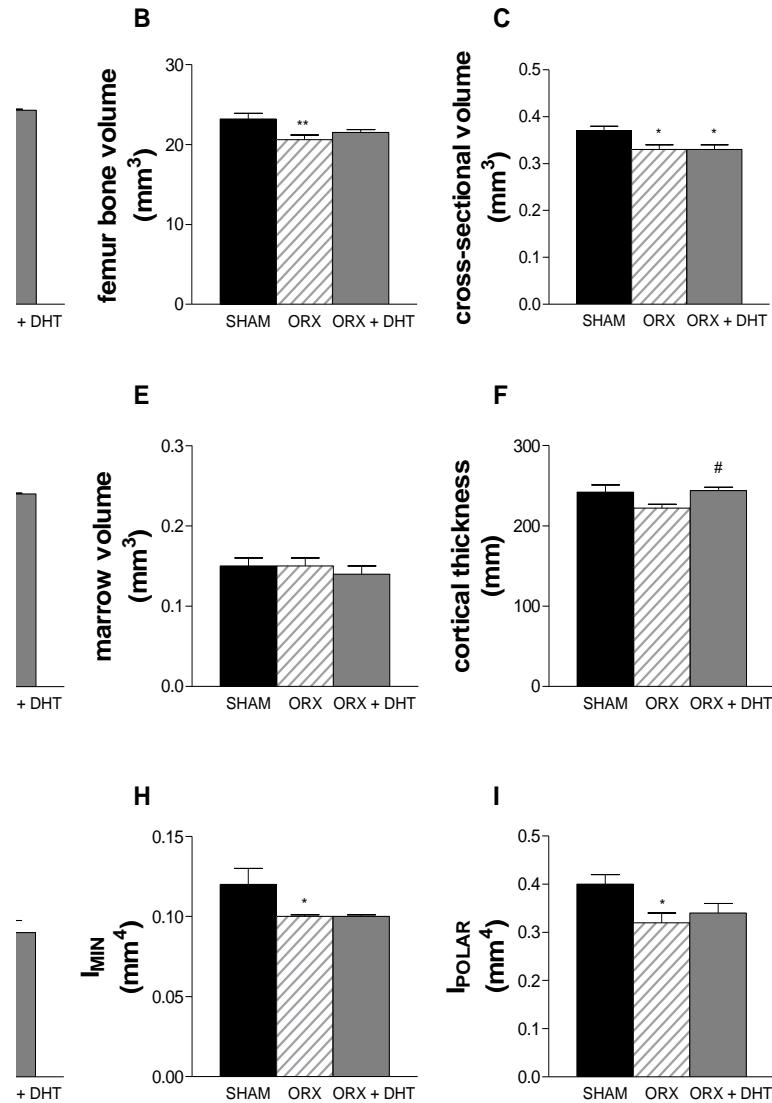
Fig. 7. Wild-type male therapeutic model µCT results from mid-shaft cortical bone. A. Femur length, B. Femur bone volume, C. cross sectional volume, D. cortical volume, E. marrow volume, F. cortical thickness, G. I_{max}, H. I_{min}, I. I_{polar}. One-way ANOVA revealed significant differences with $p < 0.05$ (A-C, F,H,I) and $p < 0.01$ (D). Tukey's multiple comparison test * $p < 0.05$, ** $p < 0.01$ vs sham controls, # $p < 0.05$ vs.ORX placebo. (n = 10-12).
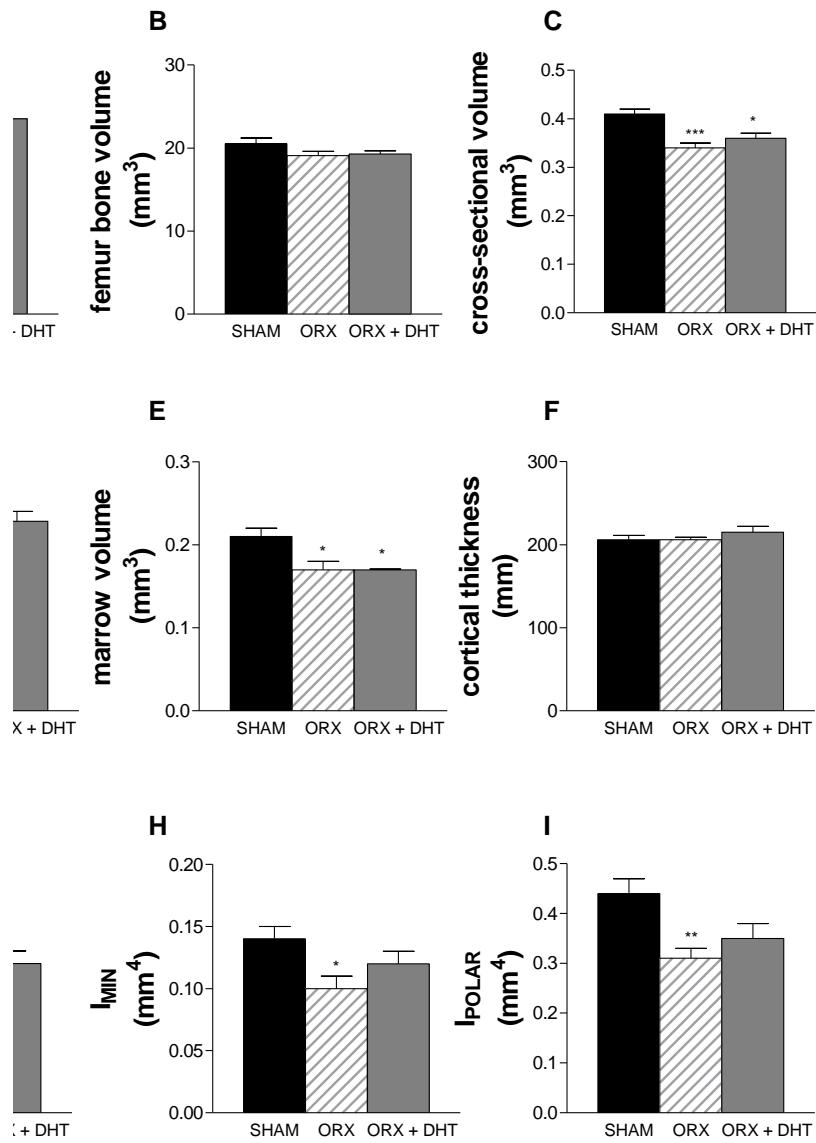
Fig. 8. AR2.3-tg male therapeutic model µCT results from mid-shaft cortical bone. A. Femur length, B. Femur bone volume, C. cross sectional volume, D. cortical volume, E. marrow volume, F. cortical thickness, G. I_{max}, H. I_{min}, I. I_{polar}. One-way ANOVA revealed significant differences of $p < 0.05$ (D,H), $p < 0.01$ (E,G,I) and $p < 0.001$ (A,C). Tukey's multiple comparison test * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. sham controls, and [#] $p < 0.05$ vs. ORX placebo. (n = 6-11).
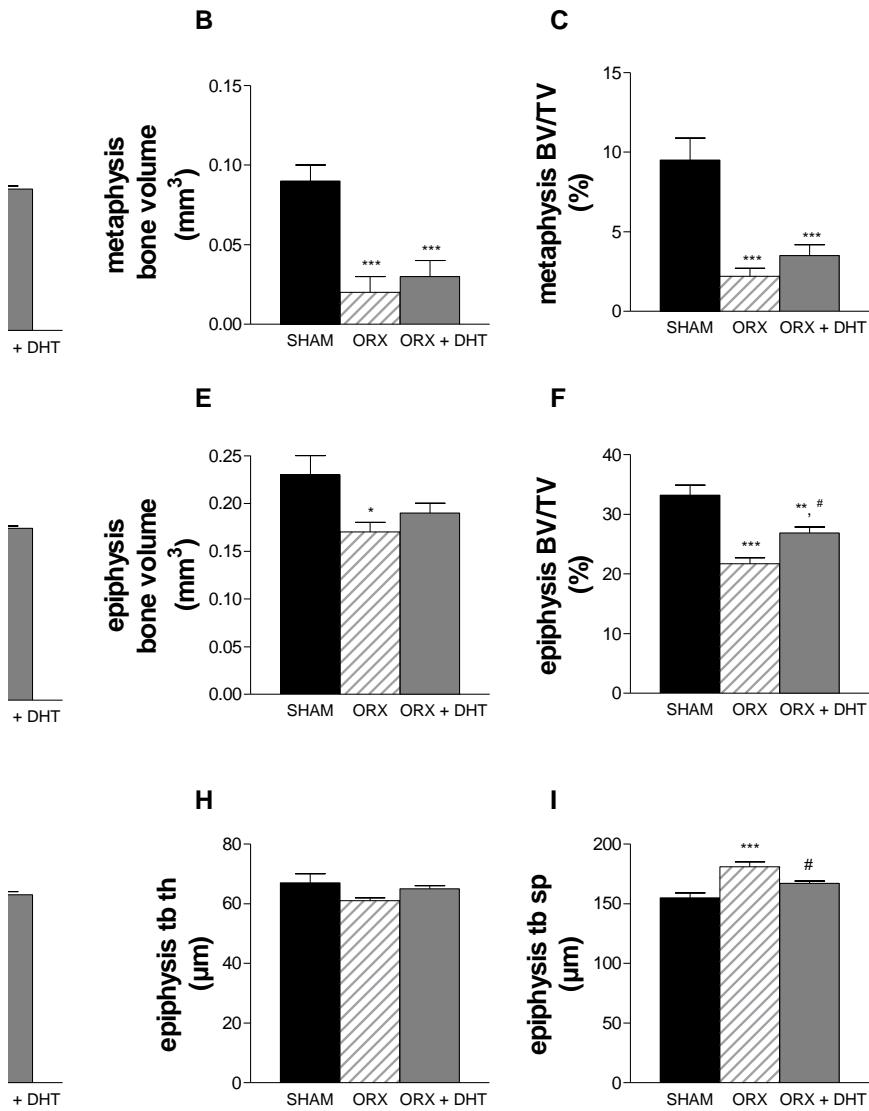
Fig. 9. Wild-type male therapeutic model µCT results from metaphyseal and epiphyseal trabecular bone. A. Metaphysis tissue volume, B. Metaphysis bone volume, C. Metaphysis BV/TV, D. Epiphysis tissue volume, E. Epiphysis bone volume, F. Epiphysis BV/TV, G. Epiphysis tb #, H. Epiphysis tb th , I. Epiphysis tb sp. One-way ANOVAs revealed significant differences of $p < 0.05$ (E,G) and $p < 0.0001$ (B,F,I). Tukey's multiple comparison test * $p < 0.05$, ** $p < 0.01$, and $p < 0.001$ vs sham controls, and [#] $p < 0.05$ vs. ORX placebo (n = 10-12).
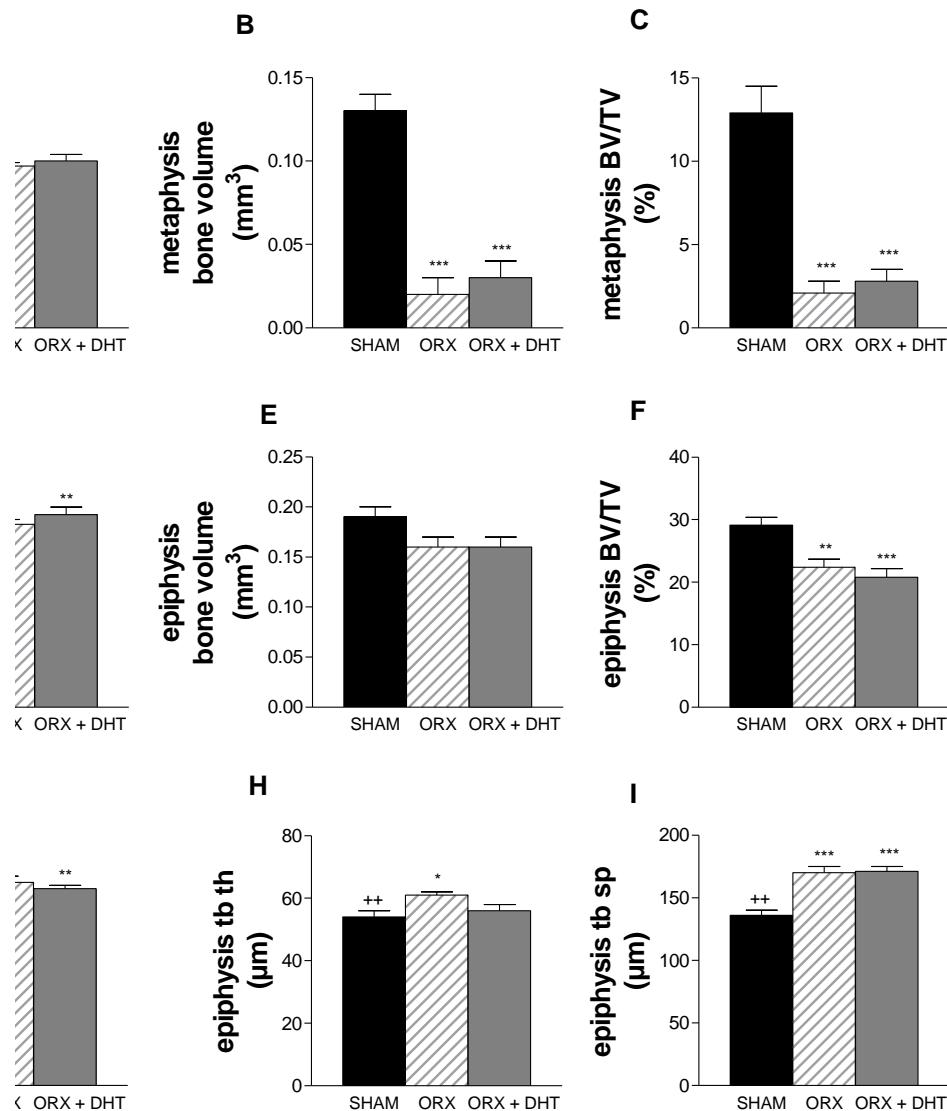
Fig. 10. AR2.3-tg male therapeutic model µCT results from metaphyseal and epiphyseal trabecular bone. A. Metaphysis tissue volume, B. Metaphysis bone volume, C. Metaphysis BV/TV, D. Epiphysis tissue volume, E. Epiphysis bone volume, F. Epiphysis BV/TV, G. Epiphysis tb #, H. Epiphysis tb th , I. Epiphysis tb sp. One-way ANOVAs revealed significant differences of $p < 0.05$ (H), $p < 0.01$ (D, G) and $p < 0.0001$ (B,C,F,I). Tukey's multiple comparison test [++] $p < 0.01$ vs. sham wild-type, [*] $p < 0.05$ and [**] $p < 0.01$ vs. tg sham controls, and [#] $p < 0.05$ vs. ORX placebo (n = 8-12).
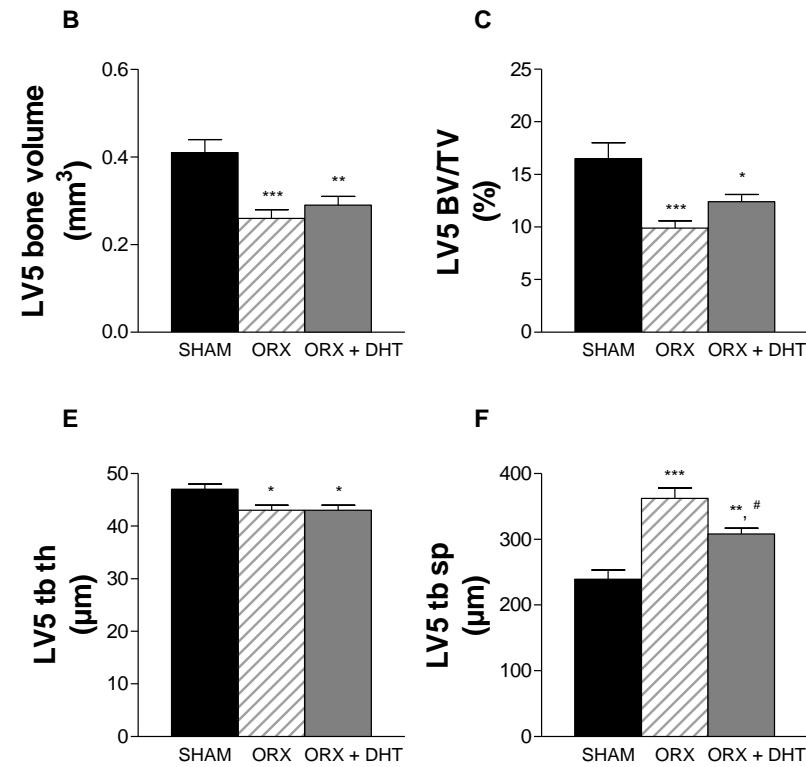
Fig. 11. Wild-type male therapeutic model µCT results from fifth lumbar vertebrae. A. LV5 tissue volume, B. LV5 bone volume, C. LV5 BV/TV, D. LV5 tb #, E. LV5 tb th, F. LV5 tb sp. One-way ANOVAs revealed significant differences of $p < 0.05$ (E) and $p < 0.0001$ (B-D, F). Tukey's multiple comparison test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. sham controls and # $p < 0.05$ vs ORX placebo (n = 13-19).
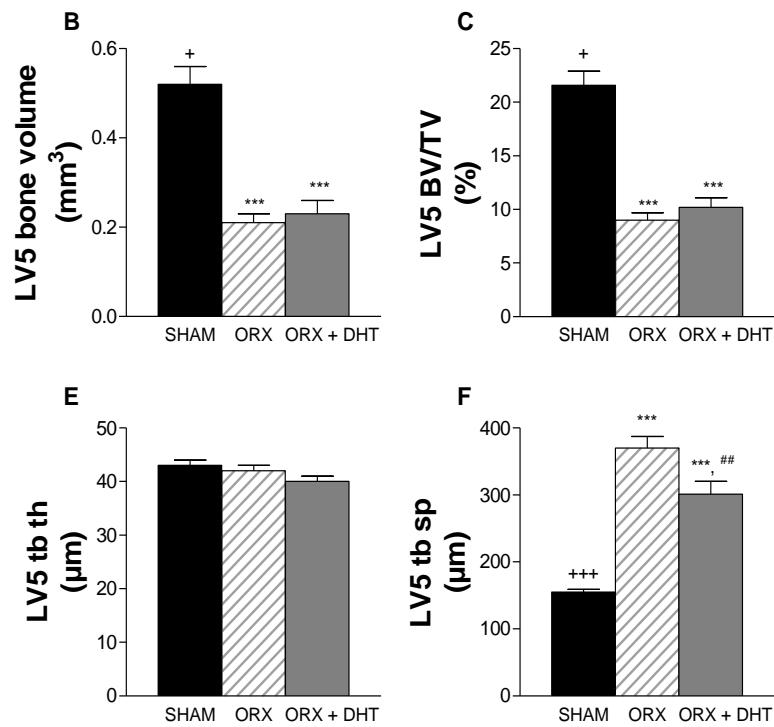
Fig. 12. AR2.3-tg male therapeutic model µCT results from fifth lumbar vertebrae. A. LV5 tissue volume, B. LV5 bone volume, C. LV5 BV/TV, D. LV5 tb #, E. LV5 tb th , F. LV5 tb sp. One-way ANOVAs revealed highly significant differences of $p < 0.0001$ (B-D, F). Tukey's multiple comparison test [+] $p < 0.05$ and [+++] $p < 0.001$ vs. wild-type sham, [***] $p < 0.001$ vs. tg sham, [#] $p < 0.05$, [##] $p < 0.01$ vs ORX placebo (n = 12).

FEMALES
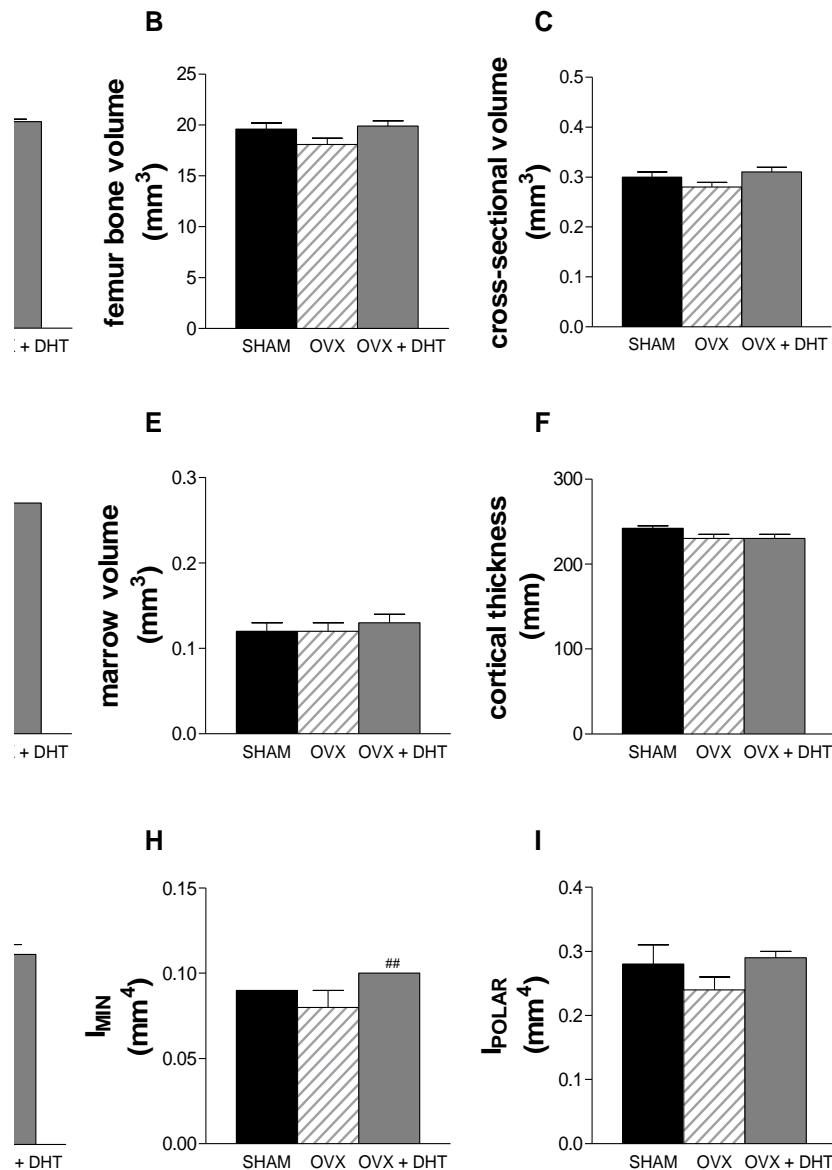
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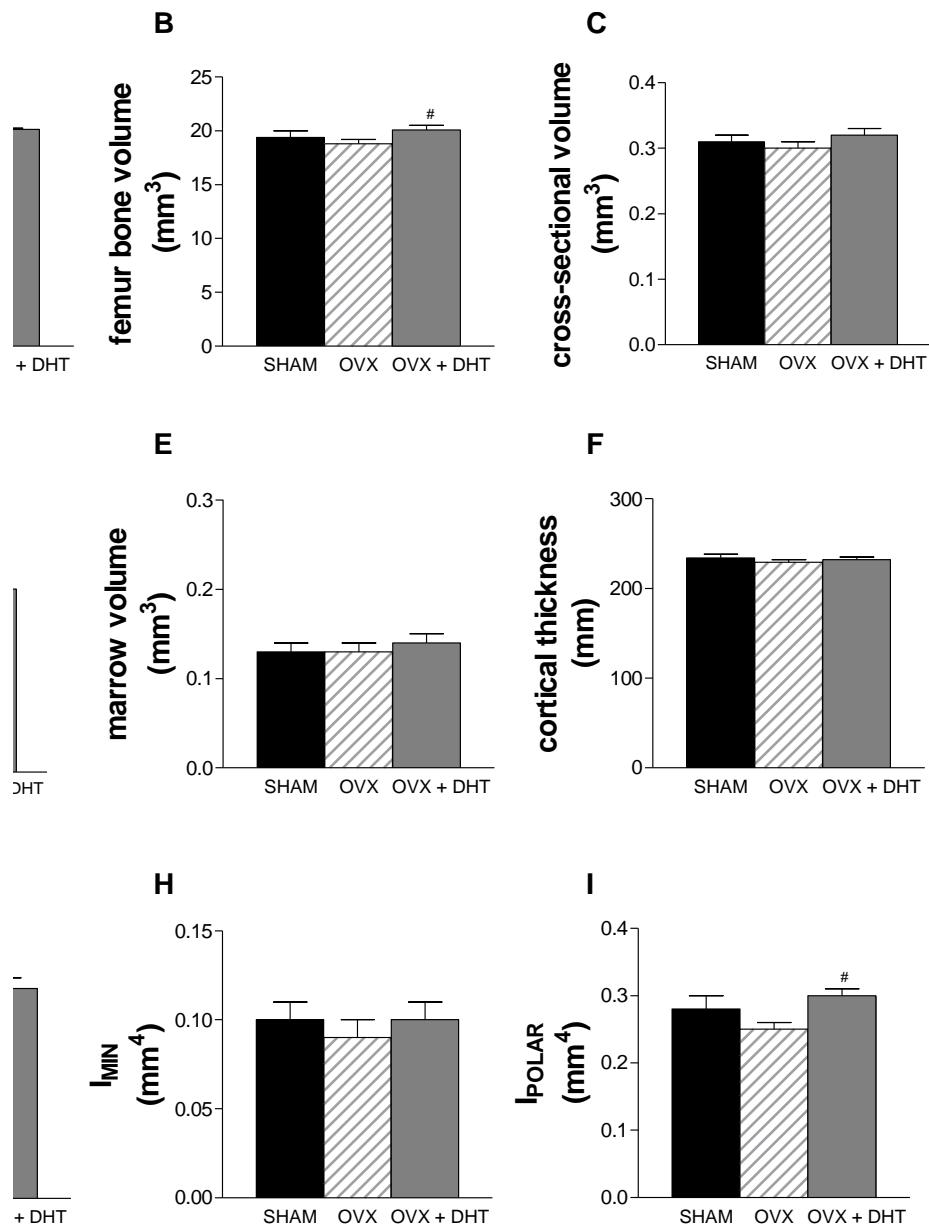
Fig. 14. AR2.3-tg female therapeutic model µCT results from mid-shaft cortical bone. A. Femur length, B. Femur bone volume, C. cross sectional volume, D. cortical volume, E. marrow volume, F. cortical thickness, G. I_{max}, H. I_{min}, I. I_{polar} Tukey's multiple comparison test $^{\#}$ $p <$ 0.05 vs OVX placebo (n = 9-16).
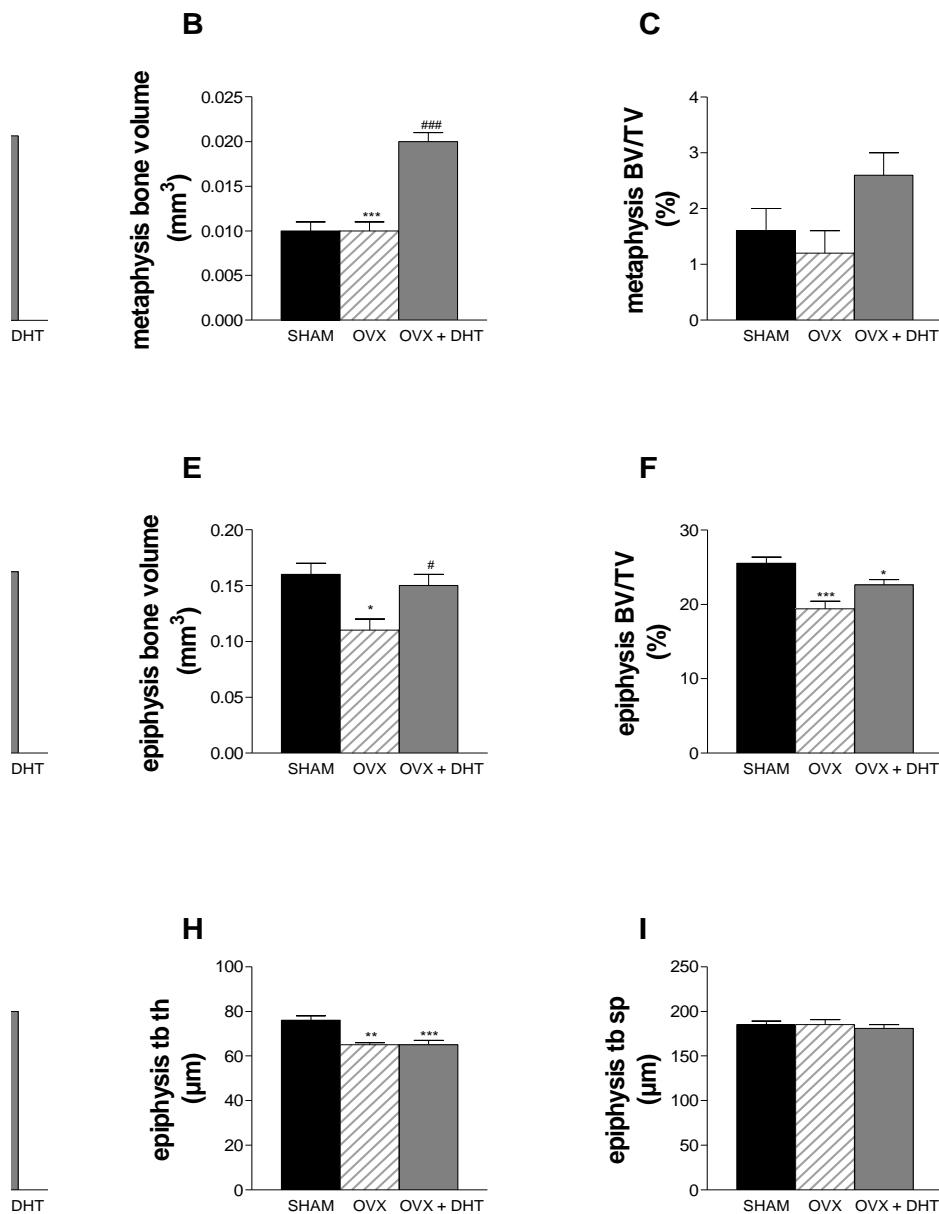
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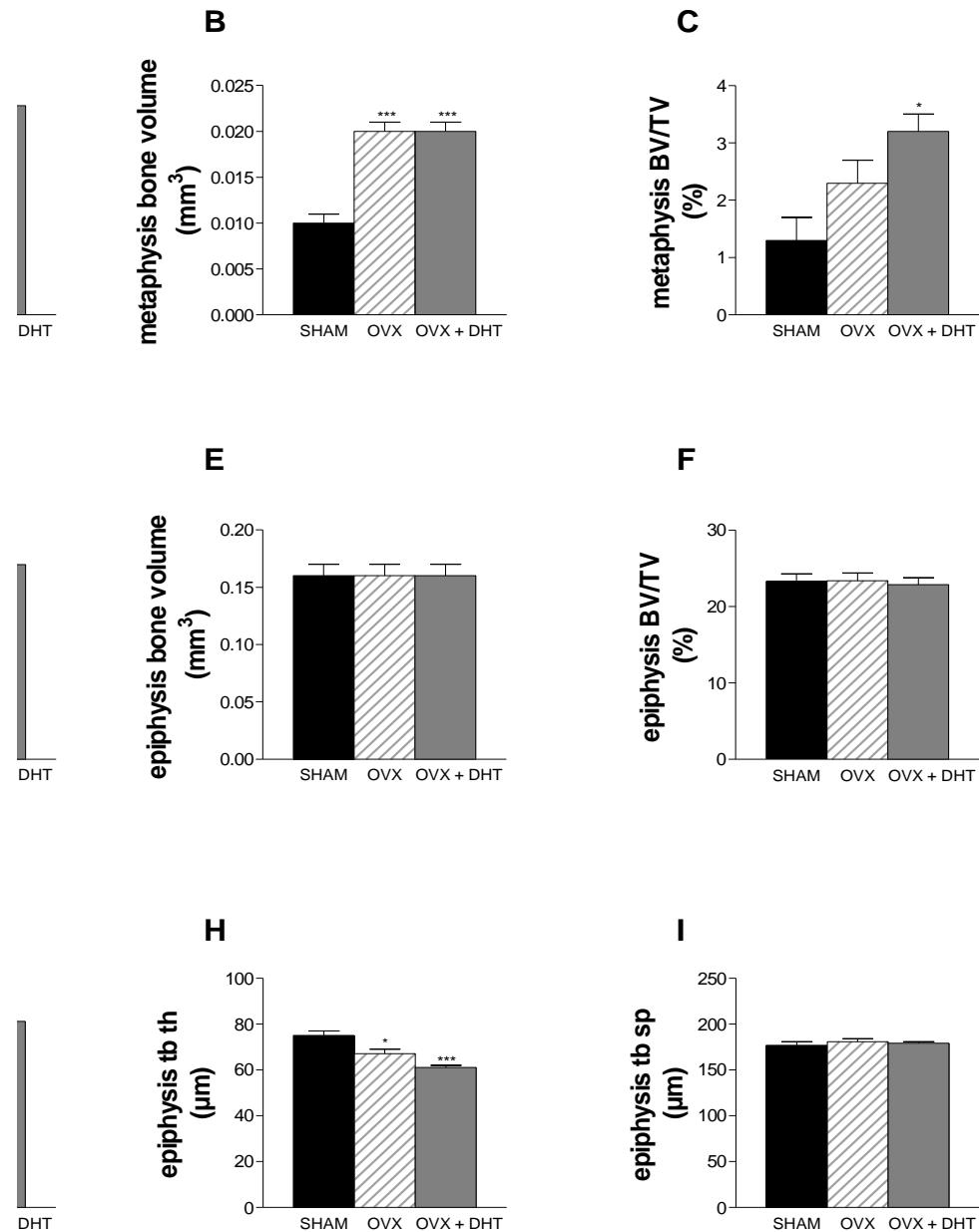
Fig. 16. AR2.3-tg female therapeutic model μCT results from metaphyseal and epiphyseal trabecular bone. A. Metaphysis tissue volume, B. Metaphysis bone volume, C. Metaphysis BV/TV, D. Epiphysis tissue volume, E. Epiphysis bone volume, F. Epiphysis BV/TV, G. Epiphysis tb #, H. Epiphysis tb th , I. Epiphysis tb sp. One-way ANOVAs revealed significant differences of $p < 0.05$ (C, G) and $p < 0.001$ (B, H). Tukey's multiple comparison test,* $p < 0.05$ and *** $p < 0.001$ vs. sham controls and [#] $p < 0.05$ vs.OVX placebo (n = 9-17).
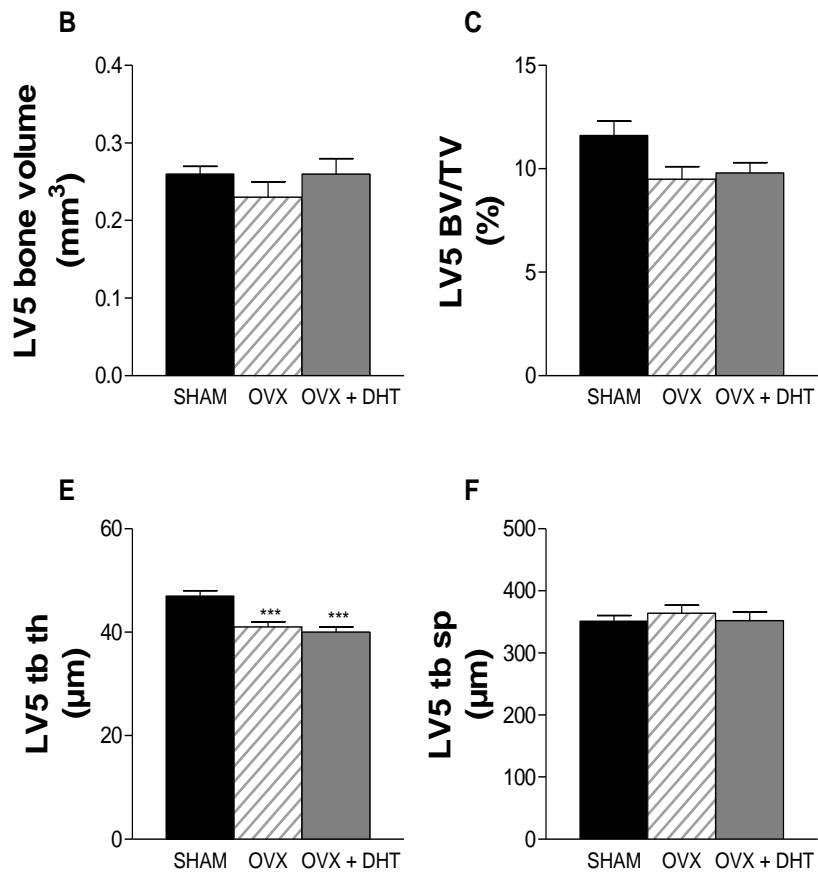
Fig. 17. Wild-type female therapeutic model µCT results from fifth lumbar vertebrae. A. LV5 tissue volume, B. LV5 bone volume, C. LV5 BV/TV, D. LV5 tb #, E. LV5 tb th , F. LV5 tb sp. One-way ANOVAs revealed significant differences of $p < 0.05$ (A) and $p < 0.001$ (E). Tukey's multiple comparison test * $p < 0.05$, *** $p < 0.001$ vs. sham controls (n = 11-19).
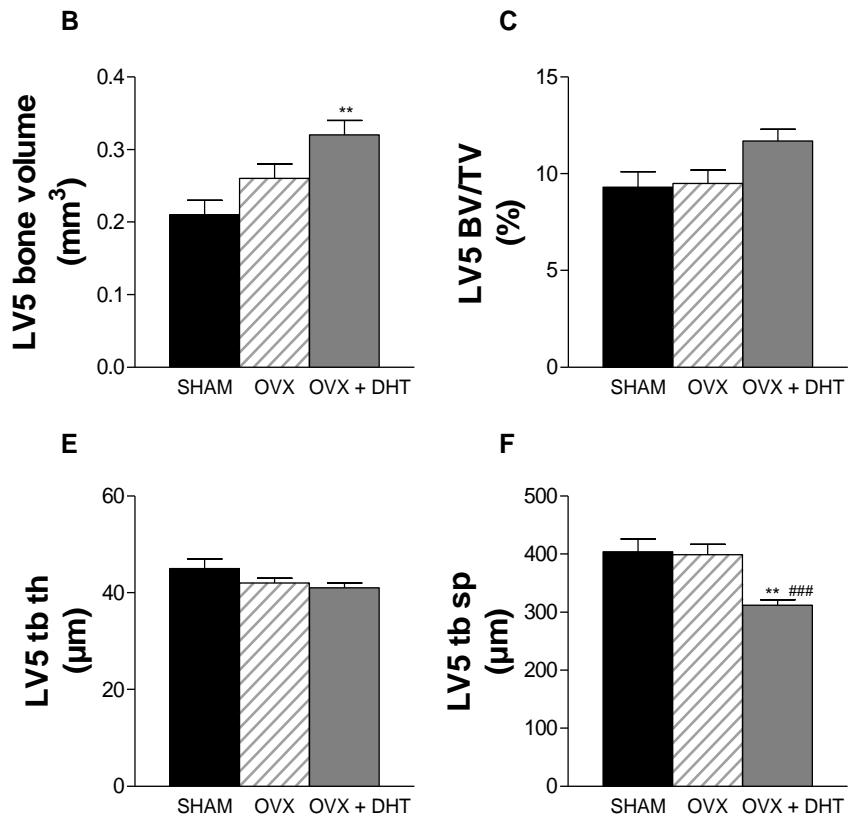
Fig. 18. AR2.3-tg female therapeutic model µCT results from fifth lumbar vertebrae. A. LV5 tissue volume, B. LV5 bone volume, C. LV5 BV/TV, D. LV5 tb #, E. LV5 tb th , F. LV5 tb sp One-way ANOVAs revealed significant differences of $p < 0.05$ (A, C), $p < 0.01$ (B) and $p < 0.001$ (D,F). Tukey's multiple comparison test * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. sham controls and ### $p < 0.001$ vs. OVX placebo (n = 9-22).

Therapeutic delayed model:

→ **Summary of key results for DXA analyses of bone for delayed treatment (low turnover); male and female wild-type vs. AR3.6-tg and AR2.3-tg mice:**

- In wild-type mice, 3.5 months in a hypogonadal state results in reduced BMD, BMC and bone area, in both males and females. Six weeks of DHT treatment beginning 2 months after gonadectomy partially restored these losses in males, but was ineffective in females.

- AR3.6-tg male mice showed a significant reduction in BMD and BMC as a result of gonadectomy, but DHT treatment failed to restore these deficits. Female AR3.6-tg mice did not lose BMD with OVX, however BMC and bone area were significantly reduced. DHT treatment had negligible effects on restoration of bone mineral in female AR3.6-tg mice.

- AR2.3-tg male mice showed a similar but less robust response to experimental manipulations as a result of gonadectomy. In both males and females there were trends for losses in BMD, BMC, and bone area with only male BMC showing significance. In this model, DHT was completely ineffective at increasing bone mineral in both males and females.

→ **Summary of key µCT results for male and female wild-type and AR2.3-tg *low* turnover (therapeutic paradigm):**

Gender Differences in WT Mice:
- **Femur:** Males have a higher total femur bone mass than females.
    - Bone length and cortical thickness did not differ between the sexes.
- **Vertebra:** There is little or no gender difference in total bone mass for LV5. Compared to females, males have a higher cancellous bone volume fraction (BV/TV). The higher BV/TV in males is due to higher trabecular number. There is no gender difference in trabecular thickness.

Effect of Gonadectomy in WT Mice
Males:
- ORX decreased total femur and total LV5 bone mass in males. The lower value in the femur was due primarily to decreases in cortical volume, cortical thickness, and potentially cross sectional volume, likely due to a reduction in periosteal deposition.
- Cancellous bone volume at all 3 sites (femur metaphysis, femur epiphysis, and LV5) was dramatically lower in mice following ORX. The resulting cancellous osteopenia was associated with lower trabecular number and thickness.
Females:
- OVX resulted in a reduction in total femur bone mass with no change in bone length and a reduction in total LV5 bone mass.
- Cancellous bone mass was lower in the femur epiphysis and LV5 following OVX. The BV/TV in femur epiphysis and LV5 following OVX was associated with lower trabecular thickness; trabecular number did not change.

Effect of DHT on gonadectomized WT mice

Males:

- Administration of DHT tended to reverse/prevent the effects of ORX on total femur bone mass and cortical bone thickness, and on whole LV5 bone mass. DHT had no effects on femur cross sectional bone volume. Regarding cancellous bone, DHT had minimal effect on proximal femur metaphysis but tended to reverse/prevent the effects of ORX on femur epiphysis and LV5.

Females

- DHT-treated OVX females do not differ from sham females in femur length, total femur bone mass, total LV5 bone mass, femur cross sectional bone volume, cortical volume, marrow volume and proximal femur epiphysis cancellous BV/TV. However, cortical thickness and lumbar vertebra cancellous BV/TV were lower and similar to OVX.

Effect of AR2.3 transgene on intact mice at 6.5 months

Males

- The transgene was associated with smaller femur length and total femur bone volume. Compared to WT, cross sectional femur volume was greater but cortical thickness was lower in TG mice. Trabecular number was higher in the distal femur epiphysis whereas trabecular thickness was lower. Compared to WT, cancellous BV/TV was higher in LV5 in the TG males. The higher BV/TV in LV5 was associated with higher trabecular number. However, trabecular thickness was slightly lower in TG compared to WT males.

Females

- There were minimal clear-cut effects of the transgene in females. LV5 cancellous BV/TV in the TG females may have been "reduced" to a magnitude comparable to OVX WT females.

Effect of gonadectomy in AR2.3-tg mice

Males

- As was the case for WT, ORX of mice expressing the transgene resulted in higher femur length, lower total LV5 total bone mass, and lower cancellous BV/TV in distal femur metaphysis and epiphysis, and in LV5. In contrast to WT, ORX of mice expressing the transgene demonstrated no change in whole femur bone volume and cortical thickness.

Females

- Following OVX, female mice expressing the transgene exhibited no cancellous bone loss at the distal femur epiphysis or lumbar vertebra.

Effect of DHT on AR2.3-tg gonadectomized mice

Males

- Compared to WT, ORX mice expressing the transgene exhibited a lower response, or no response, to DHT for the various endpoints evaluated. These consisted of: bone length, distal femur epiphysis cancellous BV/TV, femur cortical thickness, total LV5 bone volume and LV5 cancellous BV/TV.

Females

- DHT <u>may</u> have been associated with increases in total femur bone volume and LV5 cancellous BV/TV in OVX mice expressing the AR2.3 transgene.

Prevention of bone loss (in high turnover/high resorption model): In this section, we present both µCT and DXA analysis in a model of high turnover, designed for potential prevention of hypogonadal bone loss in adults. This paradigm was designed to optimize characterization of the anti-catabolic response to androgen signaling. Male and female mice were castrated at ~5 months with immediate steroid pellet replacement. DHT was delivered for ~6 weeks, and mice were then evaluated for changes in bone mineral by DXA using a mouse PIXImus2 densitometer to determine total body BMD and BMC and bone area. The µCT analysis employed a Scanco µCT40 scanner to determine cortical thickness of the femoral midshaft (20-µm voxel size) and cancellous bone volume (16-µm voxel size) in the distal femoral metaphysis and fifth LV, as described above. Analysis is now complete for male wild-type and AR2.3-tg mice (Figs. 25-30).

Immediate replacement prevention model:

→ **Summary of key results for DXA analyses of bone for immediate treatment (high turnover); male and female wild-type vs. AR3.6-tg and AR2.3-tg mice:**

- In wild-type male mice, 6 weeks in a hypogonadal state results in a trend for loss of BMD and BMC, but not bone area and DHT treatment may have slightly improved these measures. In females, BMC and bone area was significantly reduced following OVX, but BMD was not. DHT replacement showed minor improvements in bone mineral in either sex.

- Both male and female AR3.6-tg male showed significant reductions in bone mineral measures as a result of gonadectomy, and DHT treatment was effective for the prevention of bone mineral loss in male but not female mice.

- AR2.3-tg male mice lost significant amounts of bone mineral after ORX, and DHT was effective at prevention. In females BMD showed a trend towards loss while BMC was significantly reduced. In these mice, DHT was ineffective at preventing the loss of bone mineral.

MALES
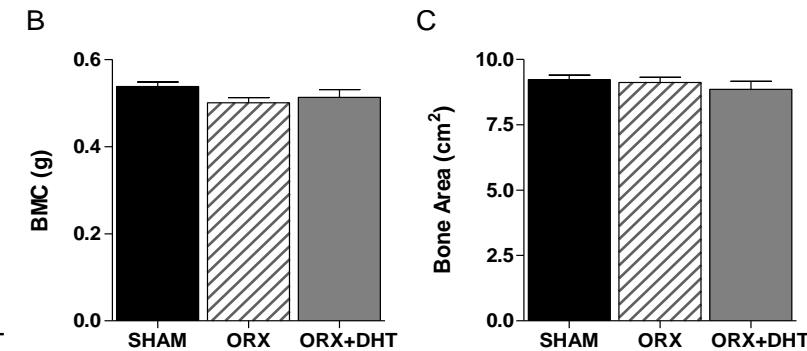
Fig. 19. Wild-type male high turnover bone mineral measures assessed by DXA. A, Whole body BMD, B, BMC and C, bone area (n = 16-29).
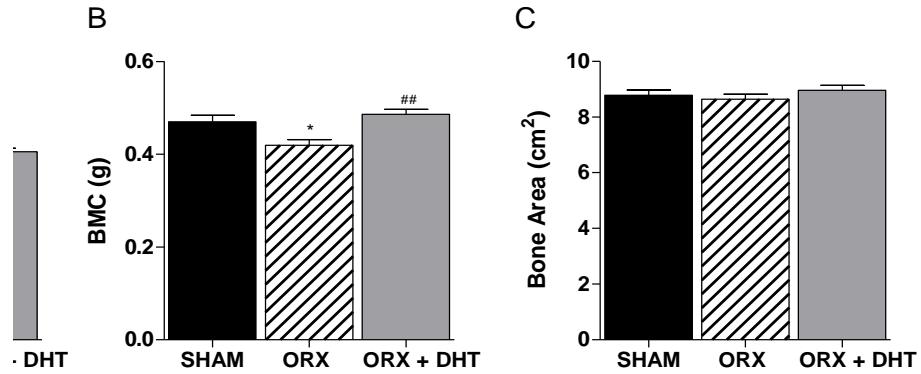
Fig. 20. AR3.6-tg male high turnover bone mineral measures assessed by DXA. A, Whole body BMD, B, BMC and C, bone area. One-way ANOVA revealed significant differences in BMD ($p < 0.001$) and BMC ($p < 0.01$). Tukey's multiple comparison test * $p < 0.05$, ** $p < 0.01$, vs. Sham controls, ## $p < 0.01$, ### $p < 0.001$ vs. ORX placebo. (n = 13-15).
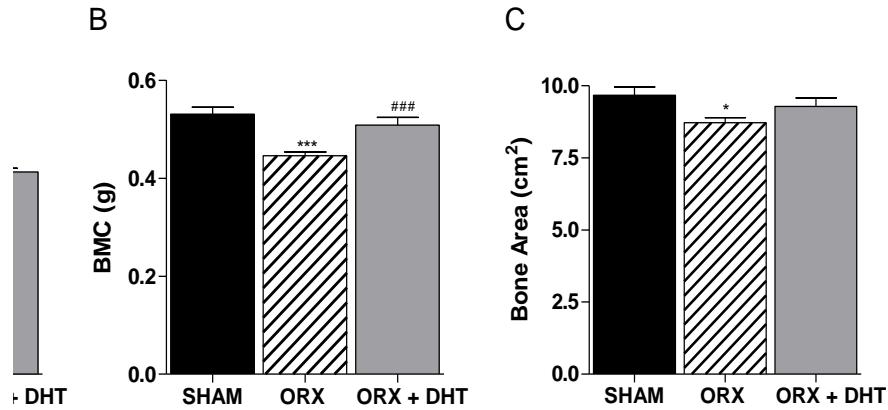
Fig. 21. AR2.3-tg male high turnover bone mineral measures assessed by DXA. A, Whole body BMD, B, BMC and C, bone area. One-way ANOVA revealed significant differences in BMD ($p < 0.05$), BMC ($p < 0.001$), and bone area ($p < 0.05$). Tukey's multiple comparison test * $p < 0.05$, *** $p < 0.001$ vs. Sham controls, # $p < 0.05$, ### $p < 0.001$ vs. ORX placebo. (n = 14-15).

FEMALES
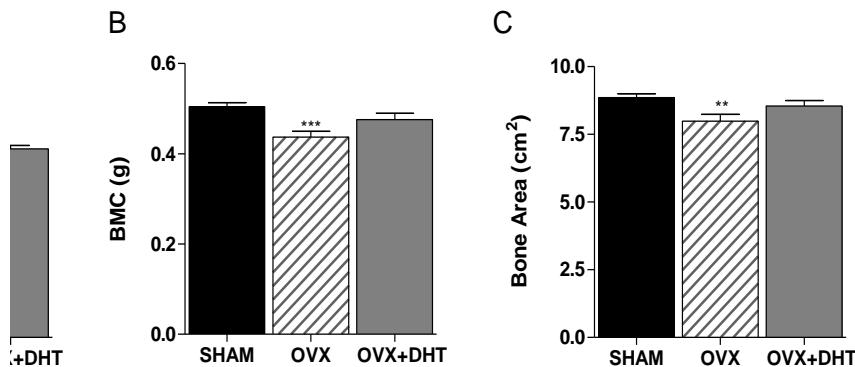
Fig. 22. Wild-type female high turnover bone mineral measures assessed by DXA. A, Whole body BMD, B, BMC and C, bone area. One way ANOVA revealed significantly reduced BMC and bone area ($p < 0.001$) following OVX. Tukey's multiple comparison test ** $p < 0.01$, *** $p < 0.001$ vs. Sham controls, (n = 24-25).
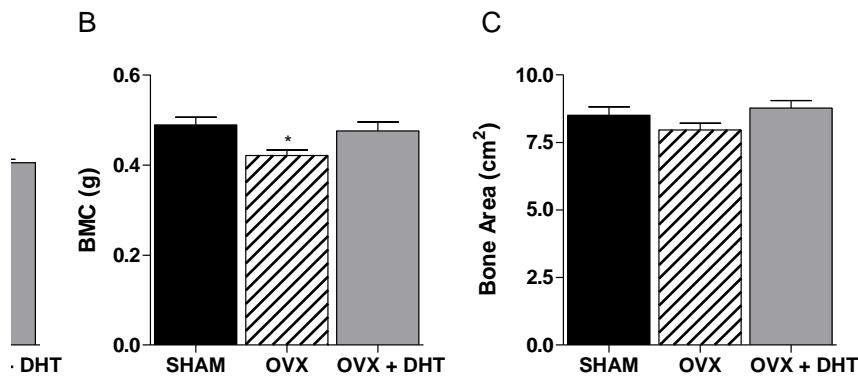
Fig. 23. AR3.6-tg female high turnover bone mineral measures assessed by DXA. A, Whole body BMD, B, BMC and C, bone area. One-way ANOVA revealed significant differences in BMD ($p < 0.01$) and BMC ($p < 0.05$). Tukey's multiple comparison test * $p < 0.05$, ** $p < 0.01$, vs. Sham controls. (n = 13-15).
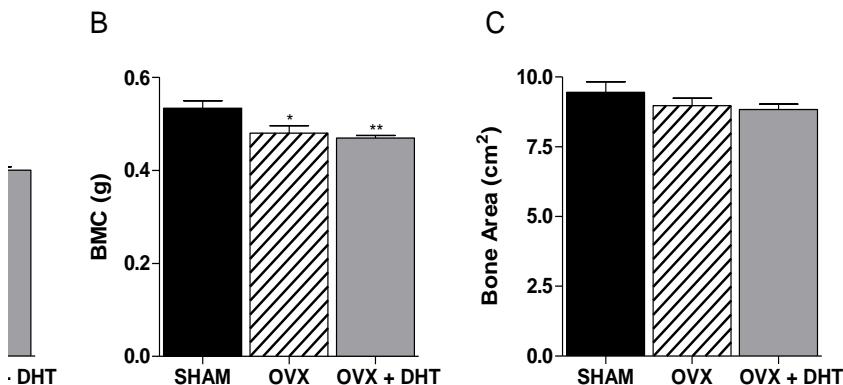
Fig. 24. AR2.3-tg female high turnover bone mineral measures assessed by DXA. A, Whole body BMD, B, BMC and C, bone area. One-way ANOVA revealed significant differences in BMD ($p < 0.05$) and BMC ($p < 0.01$). Tukey's multiple comparison test * $p < 0.05$, ** $p < 0.001$, vs. Sham controls. (n = 13-14).

MALES
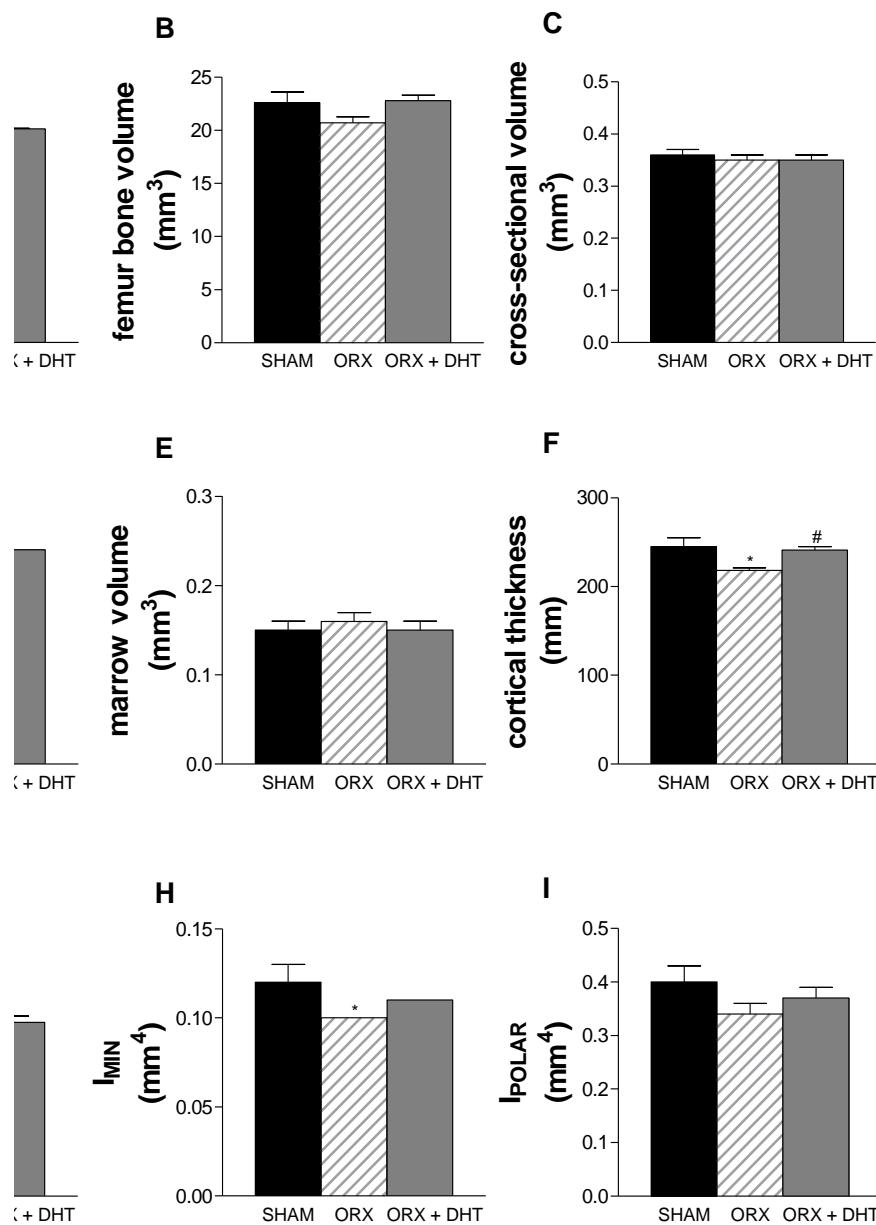
Fig. 25. Wild-type male high turnover prevention model µCT results from mid-shaft cortical bone. A. Femur length, B. Femur bone volume, C. cross sectional volume, D. cortical volume, E. marrow volume, F. cortical thickness, G. I_{max}, H. I_{min}, I. I_{polar} One-way ANOVA revealed significant differences in F $p<0.05$; Tukey's multiple comparison test * $p < 0.05$ vs. sham controls, # $p < 0.05$ vs. ORX placebo (n=8-10).

B

C

E

F

H

I
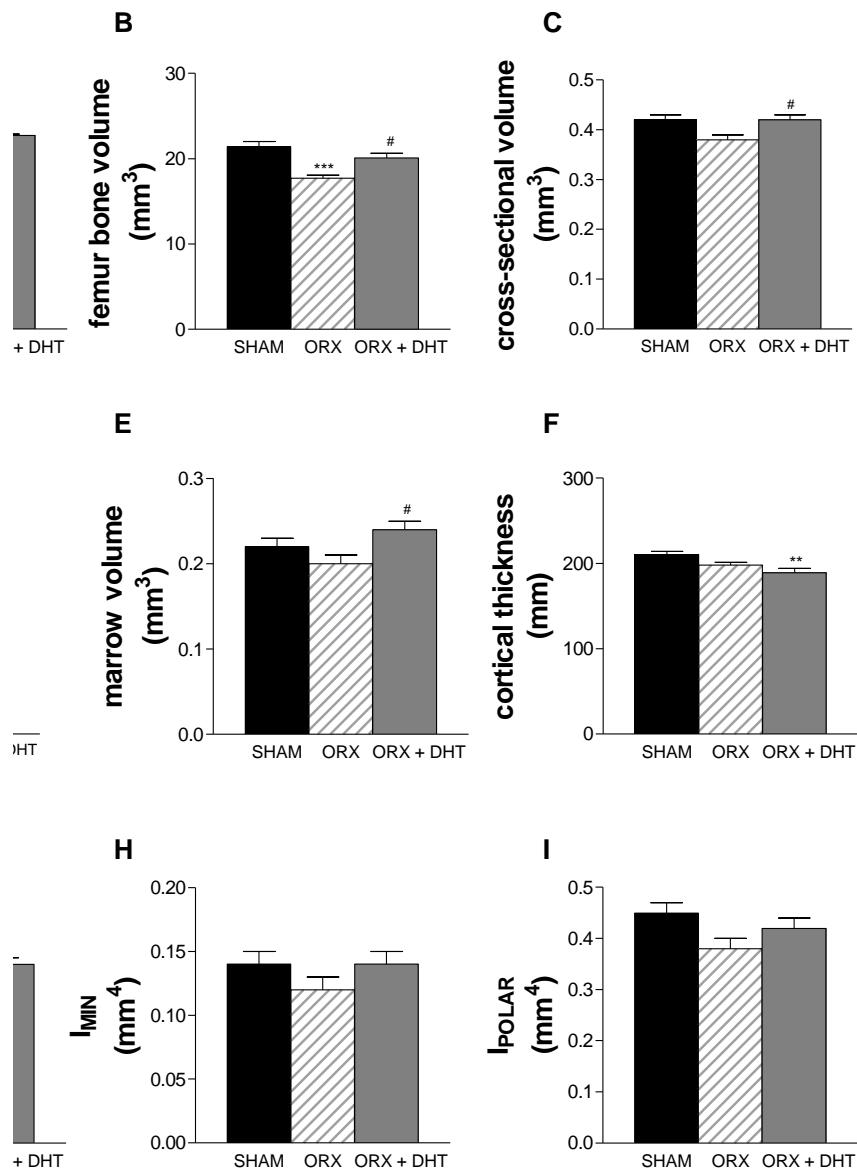
Fig. 26. AR2.3-tg male high turnover prevention model µCT results from mid-shaft cortical bone. A. Femur length, B. Femur bone volume, C. cross sectional volume, D. cortical volume, E. marrow volume, F. cortical thickness, G. I_{max}, H. I_{min}, I. I_{polar} One-way ANOVA revealed significant differences in B,F $p < 0.01$; C,E $p < 0.05$; Tukey's multiple comparison test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. sham controls, # $p < 0.05$ vs. ORX placebo (n=7-14).
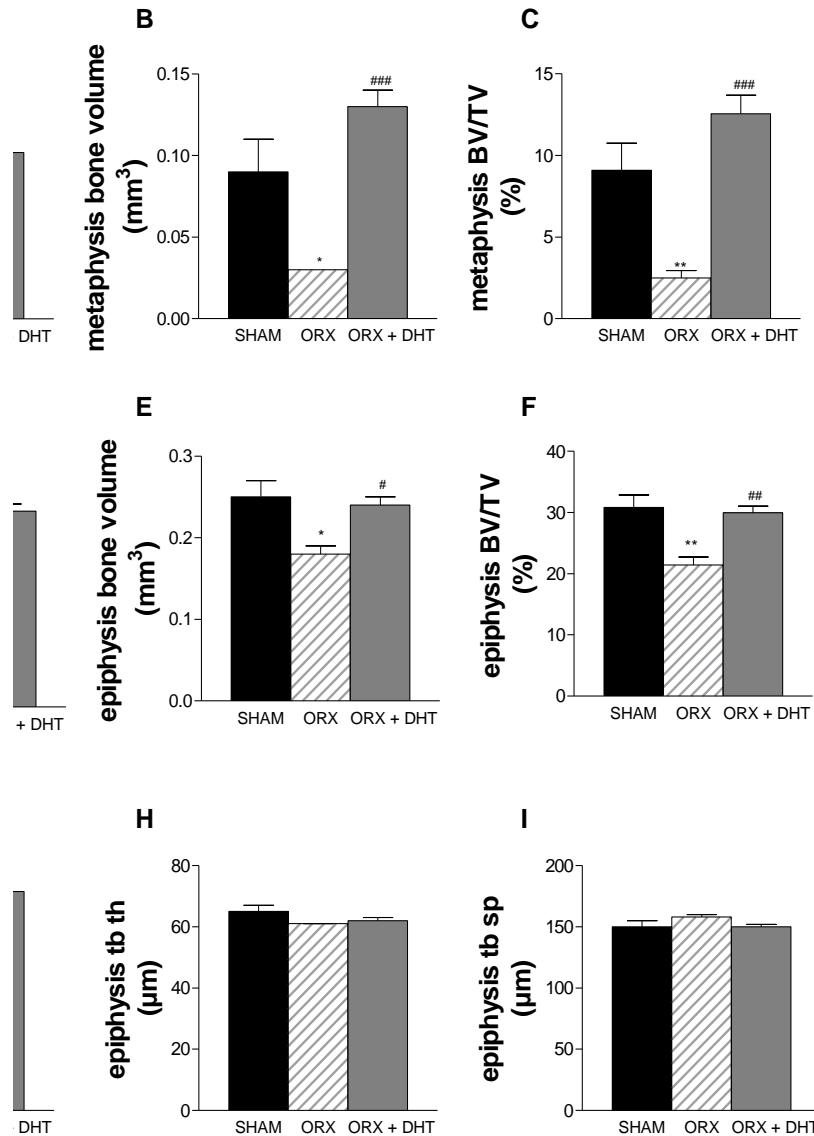
Fig. 27. Wild-type male high turnover prevention model µCT results from metaphyseal and epiphyseal trabecular bone. A. Metaphysis tissue volume, B. Metaphysis bone volume, C. Metaphysis BV/TV, D. Epiphysis tissue volume, E. Epiphysis bone volume, F. Epiphysis BV/TV, G. Epiphysis tb #, H. Epiphysis tb th , I. Epiphysis tb sp. (n = 9-13) One-way ANOVA revealed significant differences in B,C, F $p < 0.001$; E $p < 0.05$. Tukey's multiple comparison test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. sham controls, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. ORX placebo.
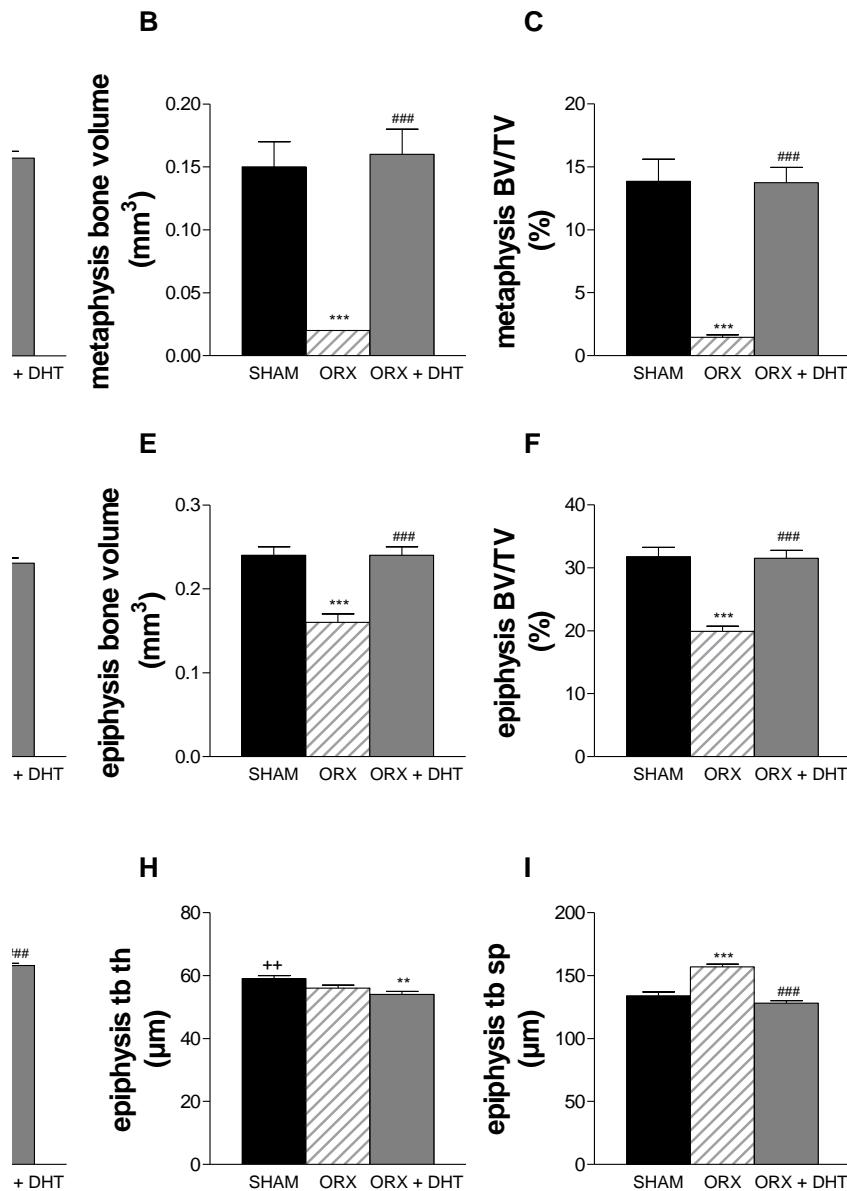
Fig. 28. AR2.3-tg male high turnover prevention model µCT results from metaphyseal and epiphyseal trabecular bone. A. Metaphysis tissue volume, B. Metaphysis bone volume, C. Metaphysis BV/TV, D. Epiphysis tissue volume, E. Epiphysis bone volume, F. Epiphysis BV/TV, G. Epiphysis tb #, H. Epiphysis tb th , I. Epiphysis tb sp. (n =11-14) One-way ANOVA revealed significant differences in B, C, E, F, G $p < 0.001$; H $p < 0.01$; D $p < 0.05$. Tukey's multiple comparison test [++] $p < 0.01$ vs WT sham?, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. sham controls, [###] $p < 0.001$ vs. ORX placebo.
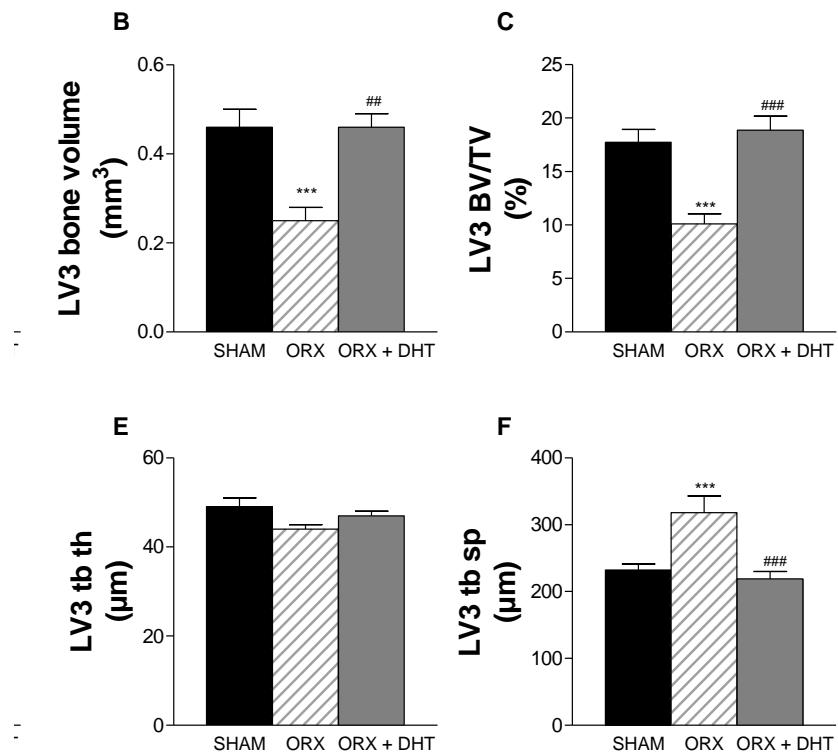
Fig. 29. Wild-type male high turnover prevention model μCT results from third lumbar vertebrae. A. LV3 tissue volume, B. LV3 bone volume, C. LV3 BV/TV, D. LV3 tb #, E. LV3 tb th , F. LV3 tb sp. (n = 9-13). One-way ANOVA revealed significant differences in B, C, D, E $p < 0.001$. Tukey's multiple comparison test *** $p < 0.001$, vs. sham control, ## $p < 0.01$, ### $p < 0.001$ vs. ORX placebo.

B

C

E

F
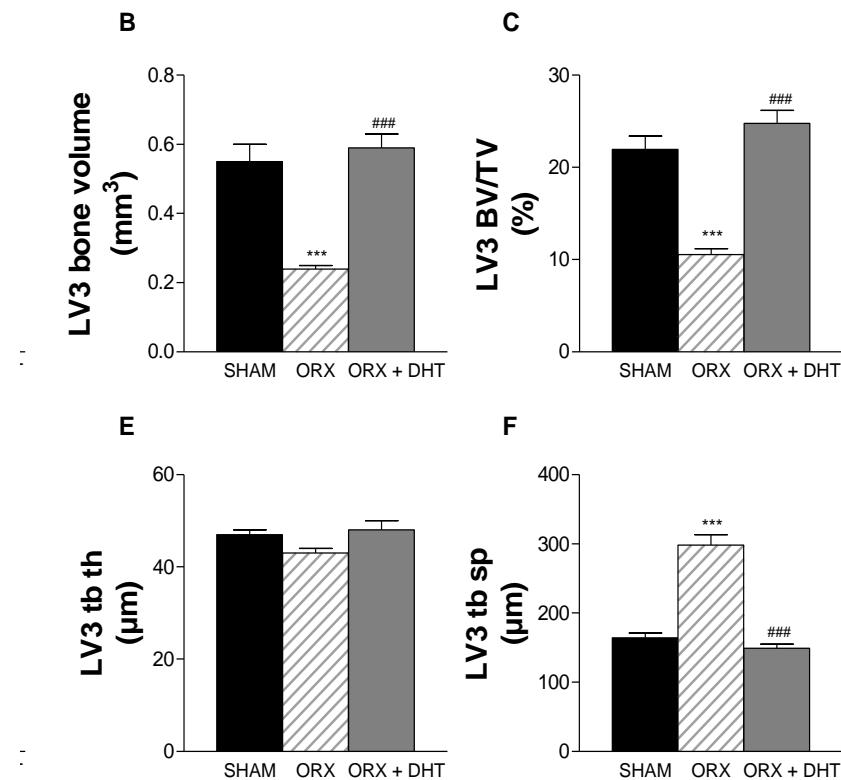
Fig. 30. AR2.3-tg male high turnover prevention model µCT results from third lumbar vertebrae. A. LV3 tissue volume, B. LV3 bone volume, C. LV3 BV/TV, D. LV3 tb #, E. LV3 tb th , F. LV3 tb sp. (n = 11-15). One-way ANOVA revealed significant differences in B, C, D, F $p < 0.001$. Tukey's multiple comparison test *** $p < 0.001$, vs. sham control, ### $p <$ 0.001 ORX placebo.

→**Summary of key results for µCT male only (female under analysis) AR2.3-tg *high* turnover (prevention paradigm):**

Effect of AR2.3 transgene on bone in intact mice at 6.5 months:
- **Femur**: Femur length was lower in AR2.3-tg mice compared to WT mice. Cross-sectional femur volume was higher but cortical thickness was lower in AR2.3-tg compared to WT mice. Thinner cortices and increased marrow volume in AR2.3-tg mice may account for the lack of genotype differences in total femur bone volume.
    - o BV/TV in the distal femur metaphysis was higher and trabecular spacing lower in AR2.3-tg mice compared to WT mice. These architectural changes were due to higher trabecular number in the AR2.3-tg animals. Differences in trabecular thickness were not detected with genotype.
    - o Differences in BV/TV in the distal femur epiphysis were not detected with genotype. However, at the architectural level, AR2.3-tg mice had slightly lower trabecular thickness and spacing but slightly higher trabecular number.
- **Lumbar Vertebra:** The transgene was associated with higher BV/TV. Compared to controls, AR2.3-tg mice had higher trabecular number and lower trabecular spacing. Differences in trabecular thickness were not detected with genotype.

Effect of gonadectomy
- **Femur**: ORX had no effect on femur length in either genotype and total bone volume in WT, but resulted in lower total femur bone volume in the AR2.3-tg mice.
    - o At the architectural level, ORX resulted in lower cortical volume and cortical thickness, irrespective of genotype. The decrease in total bone volume in mice expressing the transgene may have been due to the larger decrease in cross sectional volume observed in this genotype.
    - o Following ORX, cancellous bone volume was dramatically lower in the distal femur metaphysis and reduced in the distal femur epiphysis in both genotypes. The resulting cancellous osteopenia in both genotypes was associated with higher trabecular spacing and lower trabecular number and thickness. Although qualitatively similar, the reduction in trabecular thickness in WT may not reach statistical significance.
- **Lumbar Vertebra**: Similar to cancellous sites in the femur, ORX resulted in lower cancellous BV/TV in LV5 in both genotypes. Also, differences in the bone architectural changes associated with ORX were not detected with genotype; trabecular spacing was higher following ORX, whereas trabecular number and thickness were lower.

Effect of DHT
- **Femur**: Administration of DHT to WT mice prevented the effects of ORX on cortical bone. In mice expressing the AR2.3 transgene, DHT prevented the reduction in cross sectional volume, but not the reductions in cortical volume and cortical thickness. Interestingly, in AR2.3-tg mice, DHT increased marrow volume compared to ORX.
    - o DHT was effective in preventing most of the changes in cancellous bone mass and architecture associated with ORX in both WT and AR2.3-tg genotypes. The one exception in both genotypes was an inability to prevent the decrease in trabecular thickness in femur distal epiphysis.
- **Lumbar Vertebra**: Administration of DHT prevented the changes in cancellous bone mass and architecture in both genotypes.

GENERAL SUMMARY OF OVERALL FINDINGS FOR BONE ANALYSES:

→ **Summary of key results for DXA analyses of bone for all 4 experimental conditions (high vs low turnover; male and female wild-type vs AR3.6-tg and AR2.3-tg mice):**

In a model of *prevention in a high-turnover setting*, nonaromatizable androgen (DHT) modestly influences the non-significant changes in hypogonadal bone loss in wild-type male mice following OVX/ORX. Likely, 6 weeks is not enough time to observe significant changes. Wild-type females did demonstrate a significant reduction in BMC that was ameliorated by DHT treatment.

- In both AR-transgenic lines in the high turnover, prevention model:
  - AR3.6-transgenic male and female mice lose bone mineral following gonadectomy. Males show an anabolic response to DHT replacement, with increased BMD and BMC. In contrast, female mice generally do not improve with DHT treatment.
  - Similarly, AR2.3-transgenic male and female mice lose bone mineral following gonadectomy, but DHT treatment ameliorates these changes only in males.

In a model of *therapeutic response using a low-turnover paradigm* with established bone loss, nonaromatizable androgen (DHT) ameliorates hypogonadal bone loss in wild-type male mice following OVX/ORX but is less effective in females.

- In both AR-transgenic lines in the low turnover, therpeutic model:
  - AR3.6-transgenic mice also lose bone mineral following gonadectomy. Neither males or females show an anabolic response to DHT replacement.
  - AR2.3-transgenic male and female mice show only modest loss of bone mineral following gonadectomy, and DHT treatment has no effect in either sex.

→ **Summary of key results for µCT analyses of bone for 2 experimental conditions (high vs low turnover; male wild-type vs AR2.3-tg mice):**

In a model of *therapeutic response using a low-turnover paradigm*, nonaromatizable androgen (DHT) effectively ameliorates hypogonadal bone loss in wild-type male and female mice following ORX in cortical bone. Polar moment of inertia was reduced by ORX and was not treated by DHT, suggesting reduced biomechanical competence but was treated by DHT in female OVX mice. In females, OVX only modestly reduced cortical bone measures and DHT was effective for therapy. Cancellous bone was dramatically reduced by ORX at all three sites; androgen treatment had only very modest effects to build bone. In females, OVX had a small effect to reduce cancellous bone volume; DHT was only effective at the epiphysis.

- In AR2.3-transgenic lines in the high turnover, prevention model:
  - AR2.3-transgenic mice lose cortical bone following gonadectomy, males more than females. Males show little anabolic response to DHT replacement. Female mice show little effect of OVX or response to DHT.
  - Cancellous bone was dramatically reduced by ORX in males, mirroring wild-type responses. DHT had little to no effect to restore lost bone at all three sites. There was little effect of OVX in females, but DHT may increase trabecular number in females at LV5.

In a model of *prevention in a high-turnover setting*, there was small reduction in cortical bone and DHT restored cortical thickness in wild-type male mice following ORX. Strength parameters were unchanged. There was a significant loss of cancellous bone that was completely restored by DHT treatment.

- In AR2.3-transgenic lines in the low turnover, therapeutic model:

- o AR2.3-transgenic males show a response to DHT replacement, with increased whole bone and cross-sectional volume, with a loss of endosteal bone. Cancellous bone was dramatically reduced by ORX and was completely restored with DHT treatment through an increase in trabecular number (a hallmark of antiresorptive treatment).

To summarize, all animals for all four experimental groups (i.e., wild-type vs AR3.6-tg and AR2.3-tg; low vs high turnover paradigm) have undergone surgery and treatment, and all have been harvested. All groups have been subjected to DXA; the remaining characterization by µCT is progressing well. The delay in analysis was a consequence of lack of progress by the original subcontract site, but with the current subcontractors at OSU, progress has been dramatic over the last year. Results from female AR2.3-tg high turnover (therapeutic) analysis is nearly completed. We estimate that completion of the µCT study for all groups will require ~9 months, but we are on-target to finish for publication of results.

Combined, these data indicate that systemic DHT administration positively influences bone mineral changes in both male and female wild-type controls when present during the period of hypogonadism. Neither wild-type or mice with enhanced sensitivity to androgen through AR overexpression in the stromal compartment and proliferating osteoblasts (AR3.6) are highly responsive to the positive effects of DHT on cortical bone. In the prevention high turnover model, DHT treatment was effective at inhibiting resorptive responses in both wild-type AR2.3-tg male and female mice. Combined, our results suggest that bone, in particular the osteocyte, is not a positive anabolic therapeutic target in cortical bone. Female AR-transgenic mice appear to be less sensitive to androgen administration.

1b) *Analysis of body compositional changes gonadectomized mice:*

Androgens have well characterized anabolic effects on muscle mass and strength. In the course of characterization of bone mineral changes in AR3.6-transgenic mice, we noted that male AR3.6-transgenic mice also demonstrate a body composition phenotype, with decreased fat mass but increased lean mass. We have proposed the hypothesis that this change in body composition is a consequence of AR expression in stromal precursors; pluripotential cells with the ability to form a variety of tissues including muscle, fat, bone, and cartilage as outlined in the schema for AR2.3 and AR3.6 expression patterns (Fig. 1). We have previously shown AR3.6-transgene expression in bone marrow stromal cells. With AR transactivation in male mice during development, we propose that bone marrow stromal cells respond by alteration of lineage commitment, away from the adipocyte and instead toward the myoblast to form more muscle in the AR3.6-transgenic mice. DXA analysis has been employed to characterize body compositional changes that occur in the adult after gonadecomy and DHT replacement.

The two experimental paradigms described for bone mineral analysis were also employed for body composition analysis. In this section, we have completed analysis for potential anabolic (increased lean mass) responses in the immediate prevention vs. the delayed therapeutic model. DHT was delivered for the final 6 weeks to mice that were gonadectomized at ~3 months or ~5 months of age, and mice were then evaluated for changes in body composition by DXA. Results for the complete final analysis are shown, first with the immediate replacement paradigm and then followed by the delayed replacement after loss of lean mass in Figs. 31-36; Figs. 37-42.

MALES

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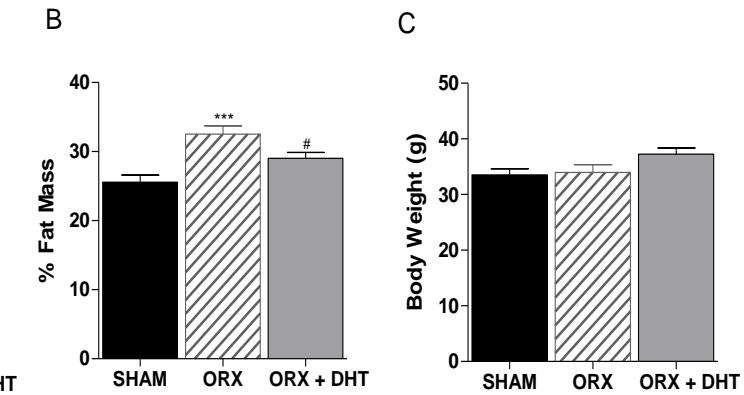
Fig. 31. Wild-type male DELAYED model body composition analysis assessed by DXA. A, % lean mass, B, % fat mass and C, body weight. *** $p < 0.001$ vs. Sham controls, # $p < 0.05$ vs. ORX placebo (n = 28-33)

B

C
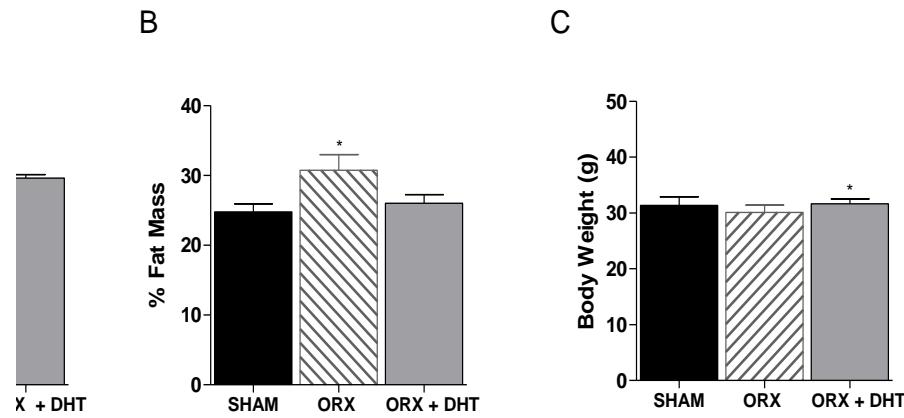
Fig. 32. AR3.6-tg male DELAYED model body composition analysis assessed by DXA. A, % lean mass, B, % fat mass, and C, body weight. One-way ANOVA revealed significant differences in lean and fat mass following ORX ($p < 0.05$). Tukey's multiple comparison test * $p < 0.05$ vs. Sham controls (n = 10-11).

B

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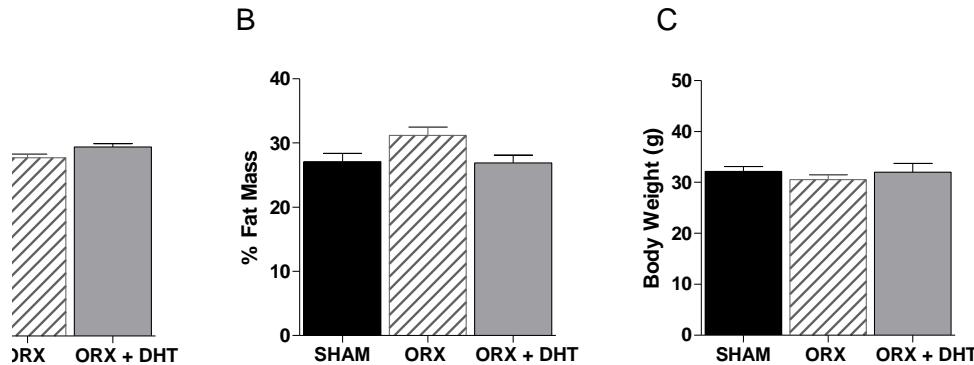
Fig. 33. AR2.3-tg male DELAYED model body composition analysis assessed by DXA. A, % lean mass, B, % fat mass, and C, body weight (n = 10-12).

FEMALES

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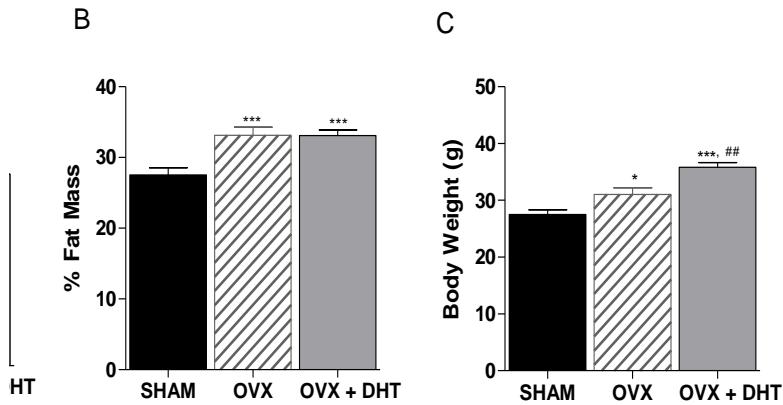
Fig. 34. Wild-type female DELAYED model body composition analysis assessed by DXA. A, % lean mass, B, % fat mass and C, body weight. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Sham controls, ## $p < 0.01$ vs. OVX placebo (n = 29-38).

B

C
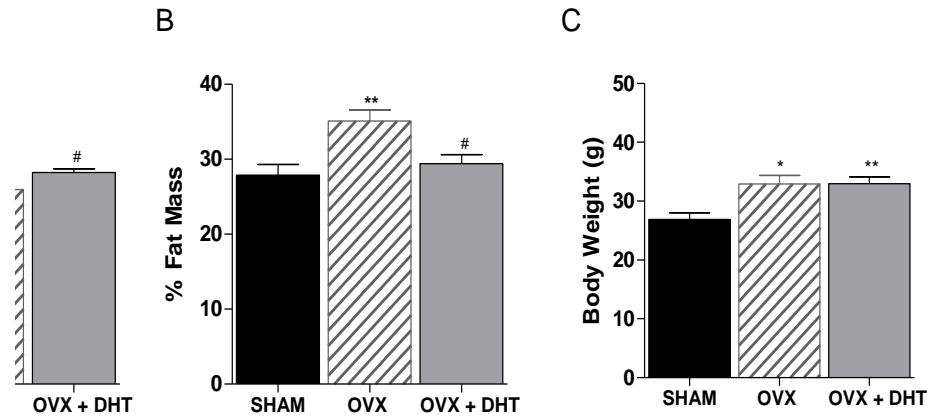
Fig. 35. AR3.6-tg female DELAYED model body composition analysis assessed by DXA. A, % lean mass, B, % fat mass, and C, body weight. One-way ANOVA revealed significant differences in lean mass, fat mass, and body weight following OVX ($p < 0.01$). Tukey's multiple comparison test * $p < 0.05$, ** $p < 0.01$ vs. Sham controls and # $p < 0.05$ vs. OVX placebo (n = 12-15).

B

C
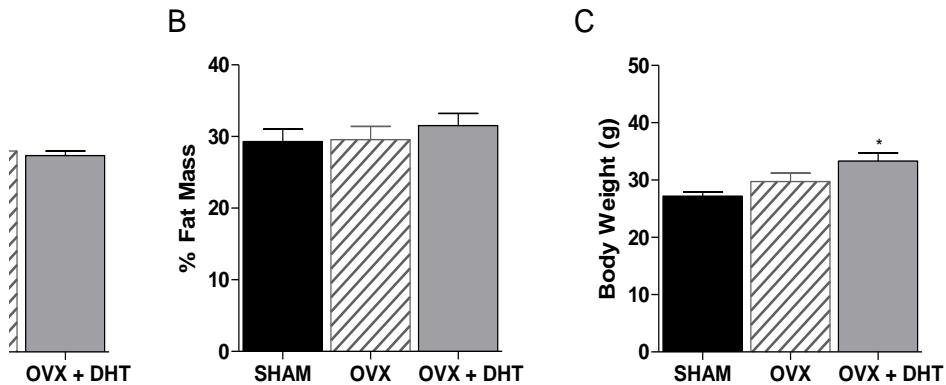
Fig. 36. AR2.3-tg female DELAYED model body composition analysis assessed by DXA. A, % lean mass, B, % fat mass, and C, body weight. * $p < 0.05$ vs. Sham controls (n = 8-13).

Gonadectomy has been shown to reduce lean mass and increase fat as a percent of body weight in humans and rodent models. Our results in the *delayed treatment therapeutic model* show that following a long period of hypogonadism with ORX, wild-type males showed reduced lean mass ($p < 0.001$) and increased fat mass ($p < 0.001$), and no change in body weight (Fig. 31 upper panels). In this setting DHT treatment was be beneficial. With DHT treatment, ORX males increased lean mass and reduced fat mass (both $p < 0.05$ vs. ORX alone) Thus, DHT administration resulted in improvements in, or prevention of, body compositional alterations. We obtained similar results in wild-type females (Fig. 34 upper panels), with a significant loss in lean mass ($p < 0.01$) and increased fat ($p < 0.001$) with OVX. Body weight was increased by OVX ($p < 0.05$). In contrast to wild-type males, there was no improvement/prevention with DHT. These results suggest that wild-type females may not be as responsive to androgen treatment as an anabolic strategy to increase lean mass.

In AR3.6-transgenic mice (Fig. 32 middle panels), gonadectomy in males resulted in a significant decrease in lean mass ($p < 0.05$). DHT replacement increased lean mass back to Sham control levels. Fat mass increased with ORX ($p < 0.05$), and DHT reduced fat mass back to Sham control levels. Body weight decreased slightly with ORX, but this was reversed after DHT replacement. Thus, male AR3.6-tg mice are more sensitive to DHT replacement to restore changes in body composition). Female patterns were similar to male (Fig. 35 middle panels). Body weight increased with OVX ($p < 0.05$) and increased further with DHT treatment ($p < 0.001$ vs. sham control; $p < 0.01$ vs. OVX). Combined, these results are consistent with AR transgene overexpression in stem cells with altered body composition as a consequence of androgen signaling.

In general, AR2.3-transgenic mice were more resistant to changes (Fig. 34 lower panels), gonadectomy in males resulted in a trend to decrease lean mass and increase fat mass. DHT replacement tended to reverse these changes. Body weight decreased slightly with ORX yet tended to reverse with DHT. In female AR2.3-transgenic mice (Fig. 36 lower panels), there were no significant effects of OVX or DHT treatment. Body weight in females was increased slightly after OVX, and increased further with DHT ($p < 0.05$).

We also determined the consequences of replacement in an immediate replacement paradigm to determine the efficacy of androgen to prevent changes in body composition. This data is shown in the following figure (Fig. 37-42). Gonadectomy has been shown to reduce lean mass and increase fat as a percent of body weight in humans and rodent models. Our results in the *immediate replacement, prevention model* show that following ORX, wild-type males showed a tendency to reduce lean mass and increase fat mass, but these changes were not significant. No change in body weight was observed (Fig. 37 upper panels). In this setting DHT treatment was not beneficial. In contrast to wild-type males, females (Fig. 40 upper panels) showed decreased lean mass ($p < 0.05$), and increased fat mass (trend) and body weight ($p < 0.05$). There was slight improvement/prevention with DHT. These results suggest that wild-type females are more sensitive to OVX in terms of changes in body composition than males but tend to be as responsive to androgen treatment as an anabolic strategy to increase lean mass.

In AR3.6-transgenic mice (Fig. 38 middle panels), males again were relatively insensitive to manipulations. Gonadectomy in males did not significantly influence lean mass, fat mass or body weight. DHT replacement did not significantly alter responses. Female AR3.6-tg mice were again more sensitive than wild-type mice to DHT treatment than males (Fig. 41 middle panels). Lean mass was significantly reduced by OVS ($p < 0.05$), with a trend to increase fat mass. DHT treatment reversed these changes (($p < 0.01$ vs. OVX alone). Body weight was not

MALES

B

C
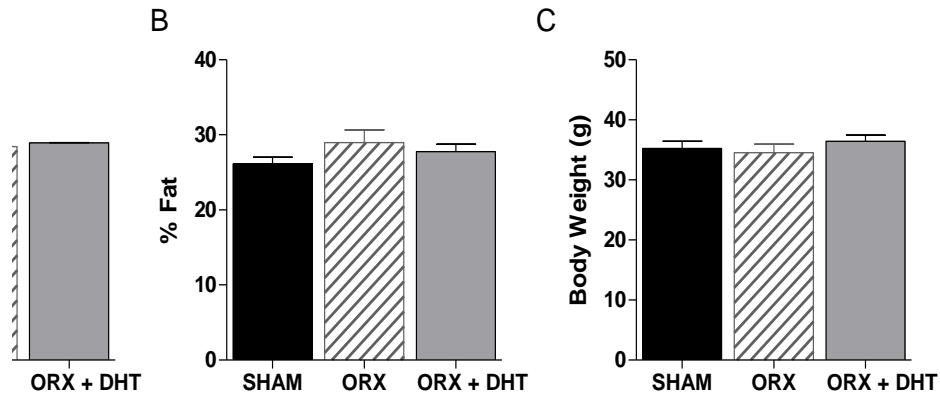
Fig. 37. Wild-type male IMMEDIATE replacement model body composition analysis assessed by DXA. A, % lean mass, B, % fat mass, and C, body weight (n = 16-29).

B

C
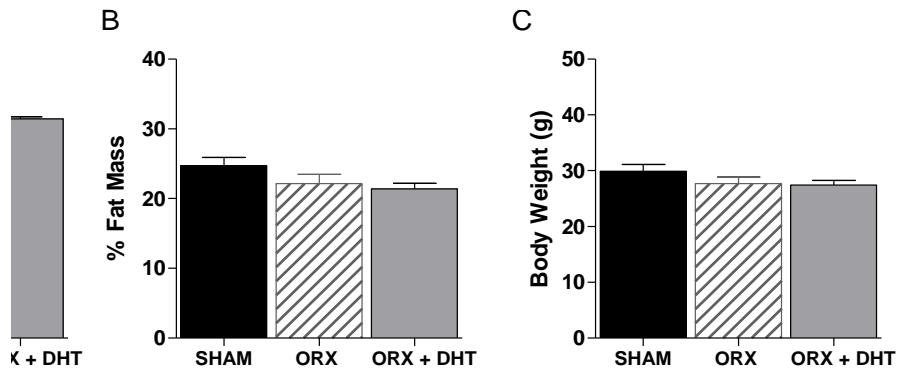
Fig. 38. AR3.6-tg male IMMEDIATE replacement model body composition analysis assessed by DXA. . A, % lean mass, B, % fat mass, and C, body weight (n = 13-15).

B

C
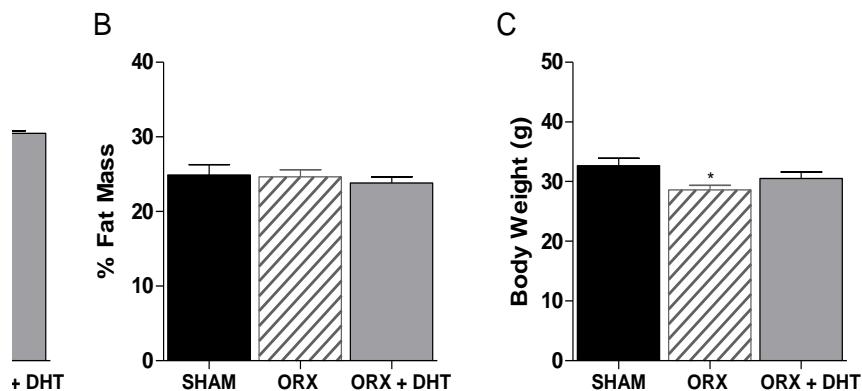
Fig. 39. AR2.3-tg male IMMEDIATE replacement model body composition analysis assessed by DXA. A, % lean mass, B, % fat mass, and C, body weight.   One-way ANOVA revealed that body weight was significantly reduced by ORX (*p* < 0.05). Tukey's multiple comparison test * *p* < 0.05 vs. Sham controls (n = 14-15).

FEMALES

B

C

% Fat Mass

Body Weight (g)

SHAM   OVX   OVX + DHT

SHAM   OVX   OVX + DHT
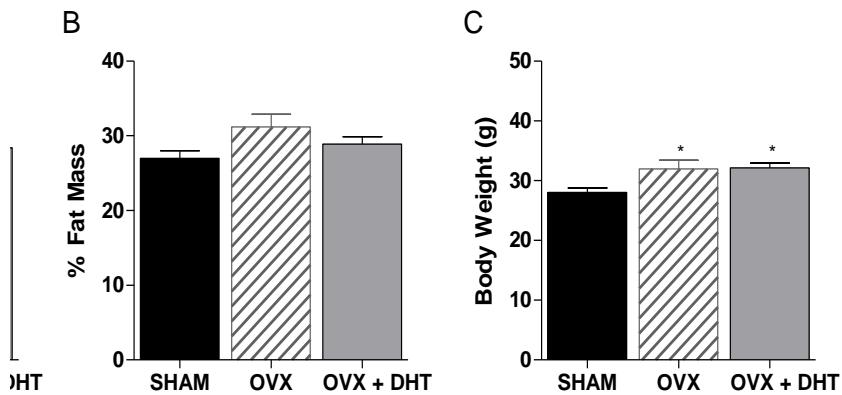
Fig. 40. Wild-type female IMMEDIATE replacement model body composition analysis assessed by DXA. A, % lean mass, B, % fat mass, and C, body weight. One-way ANOVA was significant for increased body weight ($p < 0.05$). Tukey's multiple comparison test * $p < 0.05$ vs. Sham controls (n = 23-25).

B

C

% Fat Mass

Body Weight (g)

SHAM   OVX   OVX + DHT

SHAM   OVX   OVX + DHT
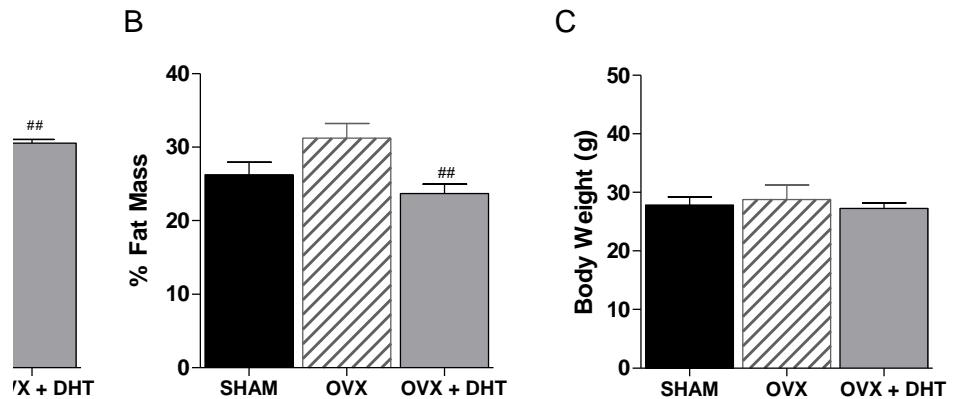
Fig. 41. AR3.6-tg female IMMEDIATE replacement model body composition analysis assessed by DXA. A, % lean mass, B, % fat mass, and C, body weight. One-way ANOVA revealed significant differences in lean mass and fat mass following OVX ($p < 0.01$). Tukey's multiple comparison test * $p < 0.05$ vs. sham controls, ## $p < 0.01$ vs. OVX placebo (n = 11-13).

B

C

% Fat Mass

Body Weight (g)

SHAM   OVX   OVX + DHT

SHAM   OVX   OVX + DHT
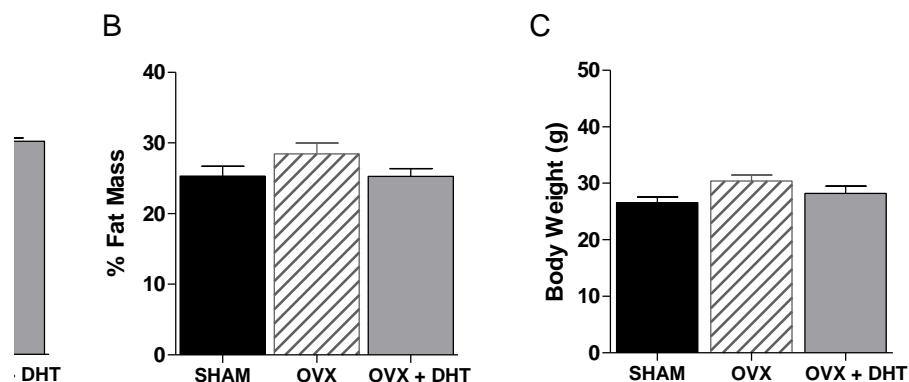
Fig. 42. AR2.3-tg female IMMEDIATE replacement model body composition analysis assessed by DXA. . A, % lean mass, B, % fat mass, and C, body weight (n = 13-14)

significantly affected. Combined, these results are again consistent with AR transgene overexpression in stem cells with females especially sensitive to manipulations after OVX.

In general, AR2.3-transgenic mice were again more resistant to changes (Fig. 39 lower panels), gonadectomy in males did not significantly affect lean or fat mass. Body weight was significantly reduced ($p < 0.05$). DHT replacement reverse this change, but did not influence lean or fat mass, similar to wild-type mice. Body weight decreased slightly with ORX yet tended to reverse with DHT. In female AR2.3-transgenic mice (Fig. 42 lower panels), OVX tended to reduce lean and increase fat mass and body weight (not significant). DHT treatment tended to reverse these modest changes. Thus, AR2.3-tg mice were less sensitive to DHT treatment than AR3.6-tg females.

SUMMARY OF FINDINGS FOR BODY COMPOSITIONAL ANALYSES:

→ **Summary of key results for DXA analyses for all 4 experimental conditions (delayed vs immediate treatment; male and female wild-type vs AR3.6-tg and AR2.3-tg mice):**

Therapeutic delayed model:

- In wild-type mice, 3.5 months in a hypogonadal state results in reduced lean mass and increased fat mass, in both males and females. A reversal was observed if DHT was present during the hypogonadal period in males but not females (with the exception of increased body weight). Wild-type female mice also showed reduced lean mass and increased fat mass but DHT treatment was ineffective in restoring these changes.

- AR3.6-tg male mice showed a significant reduction in lean mass and gain in fat as a result of gonadectomy. DHT treatment also reversed these changes. Female AR3.6-tg mice also showed decreased lean and increased fat mass with OVX. In contrast to wild-type mice, DHT treatment prevented these changes.

- AR2.3-tg male mice showed a similar but less robust response to experimental manipulations as a result of gonadectomy. DHT was modestly effective at increasing lean and decreasing fat mass in males. AR2.3 females were resistant to change after OVX, and DHT treatment had little effect, in contrast to AR3.6-tg females but similar to wild-types.

Prevention immediate replacement model:

- In wild-type mice, 6 weeks in a hypogonadal state results in reduced lean mass and increased fat mass, in both males and females. DHT treatment had little effect to prevent these changes in males while females were more responsive.

- AR3.6-tg male mice showed a significant reduction in lean mass and gain in fat as a result of gonadectomy. DHT treatment also reversed these changes. Female AR3.6-tg mice also showed decreased lean and increased fat mass with OVX. As with the therapeutic model, DHT treatment prevented these changes.

- AR2.3-tg male and female mice were relatively resistant to experimental manipulations as a result of gonadectomy. DHT was modestly effective at increasing lean and decreasing fat mass in females with no significant effects in males.

Combined, these data indicate that systemic DHT administration is effective for the prevention of the body composition changes that result from hypogonadal states in male mice, when present at the onset of the hypogonadal period. AR3.6-tg mice (both sexes) are more responsive to DHT administration. In the prevention immediate replacement model, there were significant changes in body composition in wild-type and AR3.6-tg mice but not AR2.3-tg mice. Females tended to respond to OVX more dramatically than males did to ORX. DHT was effective in preventing the changes in females. Again, AR3.6-tg mice were more responsive than wild-type or AR2.3-tg mice. These results suggest that *AR overexpression in stem cells can enhance body compositional changes in response to androgen treatment that may be beneficial in the treatment of age-related fragility*.

In addition to analysis of body compositional changes in AR-transgenic mice, we have pursued a new direction of research regarding the effects of androgen signaling in brain. With the discovery of AR overexpression in brain tissue in AR3.6-tg mice, we have evaluated the effects of enhanced androgen signaling on brain function. This work is being performed in collaboration with Dr. Patricia Hurn at OHSU (Appendix 24).

We evaluated outcomes from middle cerebral artery occlusion (MCAO) in male AR3.6-transgenic mice (Fig. 43). Transient focal cerebral ischemia was induced using reversible MCAO through the intra-luminal filament technique (8). Infarct volume was assessed in brain slices with a 1.2% solution of 2,3,5-triphenyltetrazolium chloride and fixed in 10% formalin for 24 h. Both sides of each stained coronal slice were photographed using a digital camera, and infarction was measured with digital image analysis software (SigmaScan Pro; Jandel, San Rafael, CA, USA) and integrated across all five slices. To account for the effect of edema, the infarcted volume was estimated indirectly and expressed as a percentage of the contralateral structure. The hypothesis was that enhanced androgen signaling would worsen infarct damage (8). Unexpectedly, male AR3.6-transgenic mice demonstrated significant protection from damage. We will pursue the hypothesis that changes in microglial function underlie this protective response.
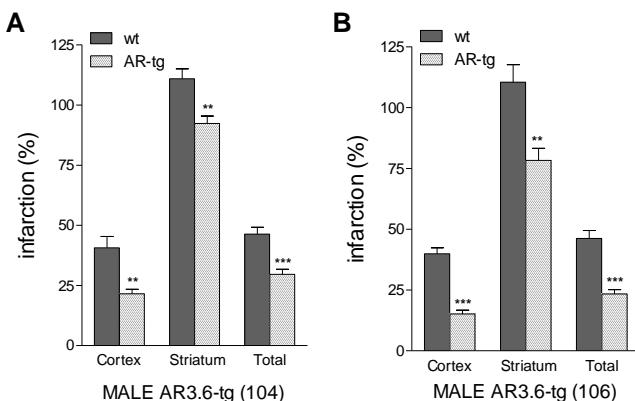
Fig. 43. Effects of enhanced androgen signaling on infarct volumes. Infarct volumes (% contralateral structure = total infarct volume of ipsilateral structure/total volume contralateral structure) were assessed in male wild-type (wt) and AR3.6-transgenic (AR-tg) male mice at 2 months of age. Mice from both independently derived transgenic families (**A**, 104 and **B**, 106) were employed. Values are mean ± SEM. **$P <$ 0.05; ***$P <$ 0.001.

Thus, aspects of the phenotype we have characterized in the AR3.6-transgenic line are consistent with reduced liability for development of metabolic syndrome and increased longevity. These include reduced body size, increased lean mass, reduced fat mass, reduced adipocyte size (manuscript in preparation) and significant protection from ischemic stroke damage (manuscript in preparation). We have thus begun a survival study to determine life-span in male AR3.6-transgenic mice. NMR determination of fat composition in the liver of 6 month old mice did not reveal a significant difference between wild-type and AR3.6-tg males; likely this will require a diet-induced obesity model to detect an expected reduction in the transgenic mice.

SECTION 2: STUDIES PROPOSED IN SPECIFIC AIM 2
Determine the importance of AR in regulating osteoclast formation and activation

Both lines of AR-transgenic mice also demonstrate a phenotype consistent with **reduced osteoclast resorptive activity** in the males. TRAP and RANKL expression is reduced, with an increase in OPG, an important negative regulator of osteoclast differentiation, survival and activation (9). In addition, the increase in trabecular bone volume with a decrease in trabecular separation observed is a hallmark of antiresorptive activity. Potential modulation of osteoclast action by DHT is incompletely characterized, although there are reports of AR expression in the osteoclast (10). The effect of androgen is undoubtedly complex, given data that androgens may inhibit levels of OPG (11, 12), although previously we (13) and others (14) have shown that androgen can stimulate OPG levels. Although androgen may be a less significant determinant of bone resorption *in vivo* than estrogen (15), this remains controversial (16). The bone phenotype that develops in a global AR null male mouse model, a high-turnover osteopenia with reduced trabecular bone volume and a stimulatory effect on osteoclast activity (17-19), also supports the importance of androgen signaling through the AR to influence resorption, and is generally opposite to the phenotype we observe with targeted AR overexpression. Interestingly, the global AR null model also develops late onset obesity (20). Finally, recent publications document that androgen reduces bone resorption of isolated osteoclasts (21), inhibits osteoclast formation stimulated by PTH (22), and may play a direct role regulating aspects of osteoclast activity in conditional AR null mice (23). Our results suggest that at least some component of inhibition of osteoclastic resorptive activity as a consequence of androgen administration is mediated indirectly through effects on mature osteoblasts and osteocytes. These findings reinforce the significance of Specific aim 2 to determine the importance of androgen in mediating osteoclast formation and activation.

2a) *Expression profiling for in vivo characterization of cortical bone derived from AR3.6-transgenic mice:*

Although our results suggest that in the AR-transgenic models, osteoclast formation is inhibited, the molecular and cellular mechanisms whereby androgen leads to this complex skeletal phenotype remain unclear and relatively unexplored. Here we sought to identify alterations in molecular signatures as well as functional consequences of increased androgen responsiveness in osteoblastic cells. We characterized differences between male AR-overexpressing mice and their wild-type counterparts in the expression patterns of osteoblast-associated genes in two distinct bone compartments; endocortical bone samples from which both periosteum and marrow elements had been removed, and periosteal bone samples that were stripped from long bone samples.

To gain a better understanding of the transcriptional basis for the inhibition of osteoblast function as a consequence of androgen signaling and to identify important pathways that are physiologically relevant, we used gene profiling analysis. Expression analysis characterized differences in long bones harvested from male mice, using the AR3.6-transgenic line as a source to identify androgen-regulated transcripts in bone because the phenotype is more robust (13, 24).

Endocortical bone samples were isolated from tibia after stripping the periosteum, flushing the marrow cavity and removal of the metaphyses, and total RNA was harvested. Gene expression was surveyed using qPCR arrays for identification of regulated transcripts, with a targeted qPCR array containing 384 genes. Analysis of expression differences using this quantitative platform is believed to be more reliable than standard hybridization approaches. Because little is known about androgen regulation of gene expression in bone, we constructed the array to contain pathways with established importance in bone development and bone remodeling, including with amplicons for genes with previously well-characterized roles in Osteoporosis, TGF-β Signaling and NFκB Signaling, and Targets of Wnt/β-catenin Signaling. This work is now published (25) (see Appendix 7).

Of the 384 array genes examined, expression of a total of 78 genes (20%) were significantly different between wild-type and AR-transgenic bones (hormone-dependent in the males). Canonical pathways were assigned using IPA analysis and the four most significantly regulated were TGFβ signaling (p = 2.2 E-12), acute phase response signaling (p = 9.5 E-08), Wnt/β-catenin signaling (p = 8.61 E-07), and IL-6 signaling (p = 1.31 E-06). The pathway that was most significantly modulated was TGFβ superfamily signaling, which would include genes for TGFβ2, BMP, Smurf1, Inhibin beta C, Smad6, Smad3, zing finger FYVE domain containing 9, Runx2, fos, jun, six3 and serpine1.

To summarize the findings, expression profile differences were characterized using array-based qPCR analysis of endocortical bone from wild-type and AR3.6-transgenic males. Bioinformatics identified the TGF-β superfamily with BMP signaling as a major pathway among those associated with bone formation to be significantly altered by androgen _in vivo_. This was confirmed in the _in vitro_ model, with stimulation of BMP signaling by addition of exogenous BMP2 abrogating androgen inhibition of differentiation. This is the **first identification of BMP signaling as an important target for androgen effects on cortical bone**.

Figure 44. Pathway analyses of androgen-regulated gene expression in cortical bone from AR-transgenic males. Significantly regulated genes expressed differentially between wild-type and AR3.6-transgenic endocortical bone samples were organized into functional groups to identify associations, based on biological processes using the PathwayArchitect module of GeneSpring GX 10. Biological processes are indicated by yellow diamonds. Genes involved in NFκB signaling are grouped.

2b) _In vivo effects on osteoclastic gene expression in male AR3.6-transgenic mice:_

To characterize biological processes that were impacted by androgen signaling in endocortical bone, we employed a second independent analysis suite with Pathway Architect software. Examination of the results revealed several notable findings. First, the most

significantly regulated GO groups were negative regulation of BMP signaling (p = 0.012), consistent with IPA analysis shown above, and regulation of mitosis (p = 0.037). We have confirmed the androgen-mediated inhibition of osteoblast proliferation *in vitro*, shown in Section 3a. Furthermore, biological categories identified included negative regulation of osteoblast differentiation, regulation of mineralization, inhibition of osteoclastogenesis/activity and regulation of chondrogenesis, all identified as biological themes targeted by androgen.

Thus, a significant biological target for androgen-mediated differences in the expression profile was **inhibition of osteoclastogenesis/activity.** Genes are clustered in boxes relative to a specific signaling cascade in Fig. 44; (see Supplemental Figure for Appendix 1, 25), with the first group of genes involved in NFκB signaling. As can be seen, inhibition of osteoclastogenesis or activity is a primary target. This data suggests that **inhibition of osteoclastogenesis and/or activity by androgen signaling *in vivo* likely involves changes in IL-6 signaling and NFκB signaling in osteoblasts,** including alteration of the RANK/OPG axis with a decrease in RANK and an increase in OPG.
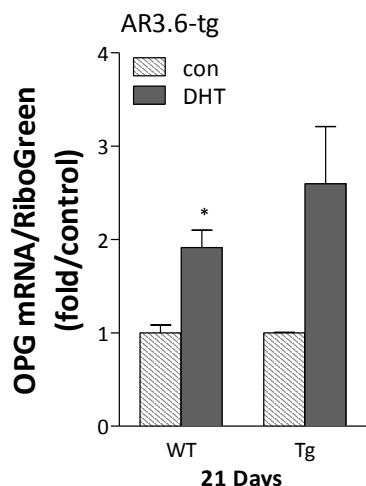
Figure 45. Real time quantitative reverse transcription PCR (qPCR) analysis of gene expression. Primary calvarial cultures derived from AR3.6-tg mice were treated continuously with 10^{-8} M DHT. After confluence, cultures were grown in differentiation medium containing ascorbic acid and β–glycerol phosphate. RNA was harvested in mineralizing cultures at d21. Data is presented as mean ± SEM. *$P < 0.05$.

To confirm this analysis, changes in expression for genes that influence osteoclastic activity were assessed by qPCR analysis. Primary cultures were treated with DHT continuously, and total RNA isolated from mature mineralizing cultures at day 21. OPG steady-state levels were reduced in wild-type and in AR3.6-transgenic cultures (Fig. 45), consistent with reduced levels in serum in AR3.6-tg (13) and AR2.3-tg (24) mice, and in endocortical samples derived from AR3.6-transgenic mice (25). Thus, enhanced androgen signaling **increased OPG expression in a cell autonomous fashion through direct effects on osteoblasts**.

SECTION 3: STUDIES PROPOSED IN SPECIFIC AIM 3
Characterize the role of androgen in regulating proliferation and differentiation in the osteoblast lineage

3a) *Completion of analysis of the effects of DHT treatment on proliferation, osteoblast differentiation and mineralization in AR3.6-tg and AR2.3-tg calvarial mouse osteoblast cultures:*

We have completed characterization of the consequences of androgen signaling on proliferation and differentiation of osteoblasts in primary cultures derived from calvaria harvested from both AR3.6-tg and AR2.3-tg mice. This work is now published (25), included as Appendix 7.

To summarize the findings, expression profile differences were characterized using array-based qPCR analysis of endocortical bone from wild-type and AR3.6-transgenic males. Bioinformatics identified the TGF-β superfamily with BMP signaling as a major pathway among those associated with bone formation to be significantly altered by androgen *in vivo*, and also revealed proliferation, osteoblast differentiation and mineralization as biological processes

affected. Stimulation of BMP signaling with exogenous BMP2 partially abrogated androgen inhibition of differentiation. In addition, **nonaromatizable DHT inhibited osteoblast proliferation, differentiation and indices of mineralization including mineral accumulation and mineralized nodule formation in normal primary cultures from wild-type and both AR-transgenic lines in a cell autonomous fashion**, consistent with *in vivo* array data and bioinformatic analyses.

Thus, **androgen action directly suppresses osteoblast function throughout the lineage**. Such detrimental effects on osteoblast function in endocortical bone would be expected to compete with the anabolic bone-formation activity seen at periosteal sites, and thus may underlie the generally disappointing results of androgen therapy. Finally, these data have therapeutic implications for the continued development of anabolic drugs for the treatment of osteoporosis, with identification important androgen-mediated signaling pathways that may negatively influence bone quality.

3b) *Characterization of androgen anabolic action by array analysis in periosteal bone to identify pathways important in osteoblastogenesis*

To gain a better understanding of the transcriptional basis for the <u>*anabolic*</u> stimulation of osteoblast function as a consequence of androgen signaling and to identify important pathways that are physiologically relevant, we used a similar gene profiling approach in isolated periosteal bone. Again, expression analysis characterized differences in long bones harvested from male mice, using the AR3.6-transgenic line as a source to identify androgen-regulated transcripts in bone because the anabolic phenotype is not present in AR2.3-transgenic males (13, 24). These studies are currently being analyzed and prepared for publication. Expression analysis revealed fewer significantly regulated transcripts in periosteal bone compared with endocortical bone. Upregulated sequences are shown in Table 1 and downregulated sequences are shown in Table 2; Fig.46 shows a volcano plot where differentially expressed genes were arranged along dimensions of biological impact (fold change) versus statistical significance (for reliability of change). The horizontal dimension shows fold change between the two groups and the vertical axis represents the *p*-value for a t-test of differences between samples.
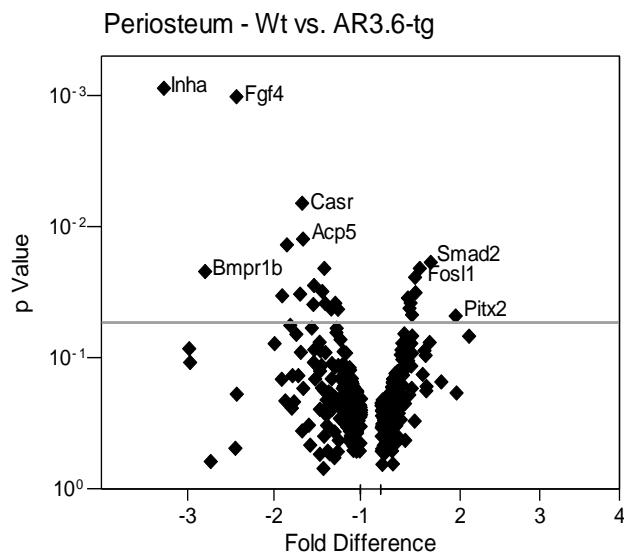
Figure 46. Gene expression differences in AR3.6-transgenic periosteal bone samples. A volcano plot of significant expression differences identified in AR3.6-transgenic bone compared with wild-type periosteal bone. This identifies sequences that are differentially expressed as a consequence of enhanced androgen signaling *in vivo*. Interestingly, there is no overlap with highly regulated sequences identified in endocortical bone.

Table 1. Upregulation of expression in array qPCR analyses in male transgenic mice from periosteal bone samples

Rank	Gene Symbol	Gene ID	Change (fold/control)	p-value
27	Six3	20473	7.0437	0.0436
29	Pitx2	18741	1.9325	0.0471
9	Smad2	17126	1.6197	0.0184
11	Fosl1	14283	1.4813	0.0204
16	Tnfsf9	21950	1.4284	0.0313
13	Mthfr	17769	1.4239	0.0239
28	Met	17295	1.3839	0.0461
20	Acvrl1	11482	1.3677	0.0375
24	Fst	14313	1.3523	0.0411
19	Itga1	109700	1.3341	0.0343

Results show genes with significant expression changes when comparing samples after global normalization. Rank number is listed in order of significant changes was significant at $p < 0.05$, calculated with respect to normalizers based on the GPR algorithm. Fold/control is shown as the expression change between transgenic and wild-type control. Analysis was from two samples in replicate representing both AR3.6-transgenic families.

Table 2. Downregulation of expression in array qPCR analyses in male transgenic mice from periosteal bone samples

Rank	Gene Symbol	Gene ID	Change (fold/control)	p-value
1	Inha	16322	-3.2852	8.63 E-4
12	Bmpr1b	12167	-2.8082	0.0216
2	Fgf4	14175	-2.4430	0.0010
18	Prkcz	18762	-1.9146	0.0330
7	Ctsk	13038	-1.8610	0.0135
17	Mgp	17313	-1.7063	0.0321
5	Casr	12374	-1.6835	0.0065
6	Acp5	11433	-1.6740	0.0122
23	Dkk1	13380	-1.5534	0.0385
14	Bmp15	12155	-1.5470	0.0276
15	Ephb3	13845	-1.4499	0.0306
10	Zfyve9	230597	-1.4257	0.0204
22	Bmp6	12161	-1.4160	0.0378
25	Tcf2	21410	-1.3460	0.0417
21	Card10	105844	-1.3005	0.0376

| 26 | Cd44 | 12505 | -1.2698 | 0.0418 |

Analysis was as described in Table 1.

Bioinformatic comparisons were used to identify important biological themes that are influenced in the periosteum, and indicate that stem cell differentiation is a target for androgen in the periosteal compartment (Fig. 47). Consistent with this analysis, the most significantly regulated biological processes were developmental process/developmental process of tissue (P = 6.49 E-10) with genes that influence development including the genes ACP5, ACVRL1, BMPR1B, CD44, CTSK, EPHB3, FGF4, FOSL1, HNF1B, ITGA1, MET, PITX2, SIX3, SMAD2; and developmental process of embryonic tissue (P = 9.96 E-05) with genes CD44, FGF4, HNF1B, PITX2, SIX3, SMAD2. Thus, enhanced androgen action in the periosteum uniquely positively affects osteoblastogeneis by modulation of stem cells.
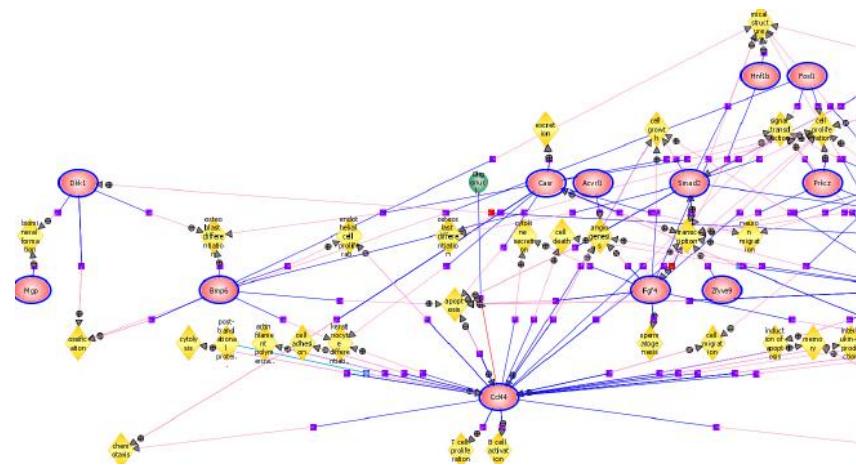
Figure 47. Pathway analyses of androgen-regulated gene expression in periosteal bone from AR-transgenic males. Significantly regulated genes expressed differentially between wild-type and AR3.6-transgenic periosteal bone samples were organized into functional groups to identify associations, based on biological processes using the PathwayArchitect module of GeneSpring GX 10. Biological processes are indicated by yellow diamonds. CD44 signaling is a central node for significantly regulated differences.

3c) *Characterization of effects of androgen in neural crest stem cells vs. mesenchymal stem cells (MSC) in vivo to contribute to androgen anabolic action to enhance osteogenesis*
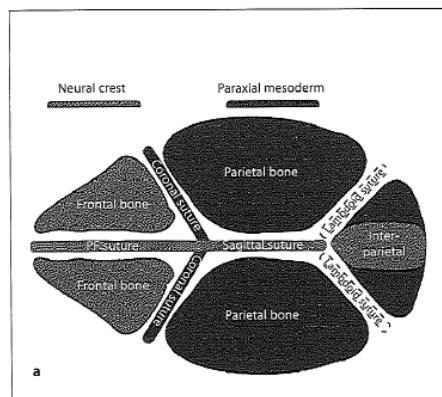
Array analysis of periosteal bone identified stem cells as a target in the in vivo situation of enhanced bone formation unique to periosteal surfaces. Because the calvaria provides a model with distinct embryological origins in different bones, we have fine-mapped the anabolic response observed in calvarial bones in male AR3.6-tg mice. As shown in the image at the left, taken from Quarto et al (26), the embryological derivation of individual bones in the calvarial cap are distinct. Thus, frontal bones are derived from neural crest stem cells, while parietal bones are derived from a mesodermal mesenchymal origin.

We had previously published that calvaria demonstrates thickening (as woven bone) in male AR3.6-tg but *not* in AR2.3-tg mice (13, 24). However, as now shown in Fig. 48, thickening of the calvarial bone structure is _not_ uniform across all calvarial bones. Saggital sections across frontal and parietal bones in calvaria harvested from male AR3.6-tg mice indicate that *only the frontal bone* demonstrates an anabolic thickening, but not the parietal bones. This result strongly suggests that androgen signaling results in

anabolic/osteogenic responses in cells derived from neural crest but not in lineages derived from mesoderm.

H-E

Von Gieson

wt   Tg
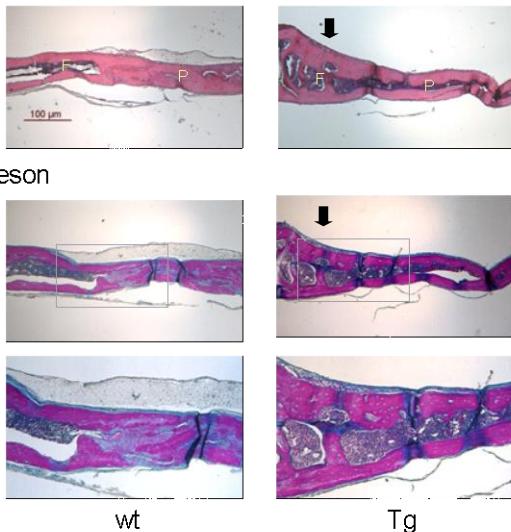
Figure 44. Histological analysis of site-specific anabolic action in calvaria from AR3.6-tg male mice. Sagittal sections of frontal (F) and parietal (P) bones from 2-month calvaria. Calvaria were harvested from wild-type (left; wt) and AR3.6-transgenic mice (right; tg) and processed. Sections were stained with H-E (hematoxylin and eosin) (upper panels) and von Gieson stain to identify newly synthesized collagen bundles (lower panels). Note the dramatically increased width of frontal bone in AR3.6 transgenic calvaria vs. parietal bones (arrow) and vs. wild-type controls.

Original magnification x4, inset x10.
Scale bar = 100 μm.

We have also evaluated transgene expression in bone marrow mesenchymal stem cells, and show high level expression. Preliminary analysis in proliferating precursors derived from frontal bone (i.e., neural crest) also show high level AR-tg expression. This result suggests that **AR signaling in neural crest results in enhanced osteoblastogenesis and bone matrix production, while AR signaling in MSC results in inhibition of the osteoblast lineage**. Since androgen increases osteoblastogenesis at the periosteal surface, we **hypothesize that periosteal osteoblasts are thus also derived from a neural crest stem cell origin**. It may therefore be advantageous to focus future development of bone anabolic therapies in neural crest stem cell lineages.

3d) *Characterization of the differentiation potential of AR3.6-transgenic bone marrow stromal cells in ex vivo culture.*

Finally, we characterized differentiation of marrow stromal cells by quantifying colony forming unit-fibroblast (CFU-F) from AR3.6-transgenic mice. CFU-F is an *in vitro* estimate of MSC precursors in the bone marrow compartment. Bone marrow stromal cells (MSC) or mesenchymal stem cells are a pluripotent population of cells found at a frequency of 0.5 %-1% in bone marrow. Depending on the signaling milieu of growth factors and hormones received, BMSC will differentiate into multiple lineages including the myoblast, osteoblast, and adipocyte. AR3.6-transgenic lines are the most informative source of stromal cells because marrow cells show high level expression of the AR-transgene in this line. These studies help to characterize the effects of androgen signaling on lineage commitment, and how androgen can influence osteogenesis.

As shown in Fig. 49, we determined whether AR overexpression influences the ability of stem cells to differentiate along a mesenchymal lineage. MSCs were isolated from wild-type and AR3.6-transgenic male mice. Femurs were harvested, stripped free of muscle, the metaphases were removed and the bone marrow was collected by centrifugation or was flushed using a syringe and 27 g needle. A single cell suspension was plated in 6-well plates at 1.5 x 10^6 cells per well for CFU-F in α-MEM containing 15% fetal calf serum. After six days in culture,
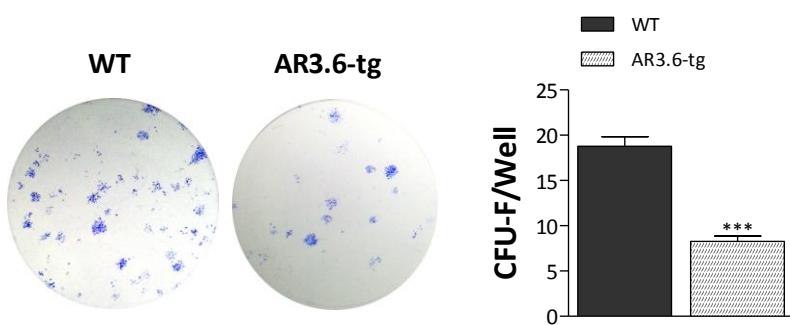
Figure 49. Analysis of CFU-F colony formation. Quantification of CFU-F numbers between wild-type and AR3.6-transgenic male mice. CFU-F is an *in vitro* estimate of MSC precursors in the bone marrow compartment. Graph at right shows significant reductions in CFU-F numbers in cultures harvested from AR3.6-tg mice. *** $P > 0.001$.

half of the media was replaced and the cells were grown for an additional 4-6 days. On day 10-12, cultures were washed, fixed with 4% paraformaldehyde and stained with Giemsa. Digital images were captured and the number of CFU-F colonies were enumerated. As can be seen, AR3.6-transgenic marrow contains fewer MSC precursors than wild-type mice. In Fig. 50, marrow cells were plated at 2.5 x 10^6 cells/well and the ability to differentiate toward the osteoblast lineage was quantified in CFU-OB numbers. Consistent with a reduction in osteogenesis observed in AR3.6-transgenic mice, there were significant reductions in total and AP+ CFU-OB numbers in cultures harvested from AR3.6-tg mice.
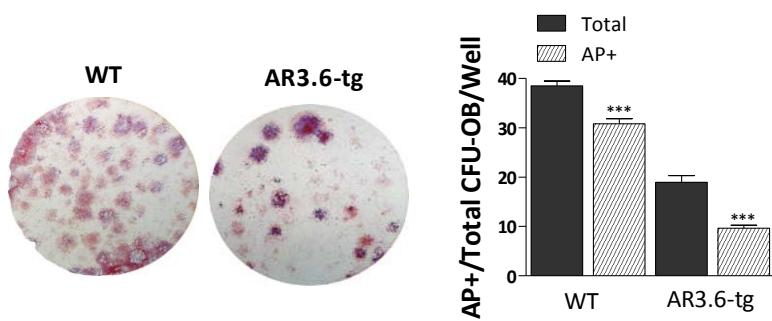
Figure 50. Analysis of alkaline phosphatase positive CFU-OB colony formation. Quantification of CFU-OB numbers between wild-type and AR3.6-transgenic male mice. Ratio between total colonies vs. alkaline phosphatase positive (AP+) colonies. Graph at right shows significant reductions in total and AP+ CFU-OB numbers in cultures harvested from AR3.6-tg mice. *** $P > 0.001$.

SUMMARY OF RESULTS FOR FINAL PROGRESS REPORT:

The specific role of the AR in maintenance of skeletal homoeostasis remains controversial, and confusion exists regarding the *in vivo* action of androgens in bone due to i) metabolism to estrogen, ii) because androgen influences many tissues in the body and iii) many months of treatment are required to observe improvement in BMD. To determine the specific physiologic relevance of androgen action in the mature osteoblast/osteocyte population in bone, mice with targeted AR overexpression in mature osteoblasts were developed to compare and contrast

with transgenic lines with AR overexpression in stromal cells and throughout the osteoblast lineage.

An important advantage of the both the AR3.6-transgenic and AR2.3-transgenic mouse models are the enhancement of androgen signaling as a consequence of increased AR abundance in likely target (tissues or cells) for androgen *in vivo*, i.e., periosteal cells and the osteoblast lineage compared to mature osteoblasts and osteocytes. These models, characterized by the absence of differences in circulating testosterone or 17ß-estradiol and studied without systemic androgen administration, thus takes advantage of increased sensitivity to androgen in distinct skeletal sites for the analysis of compartment-specific effects of androgen. At the same time, AR overexpression, rather than systemic administration, excludes action at other androgen target tissues *in vivo* including muscle and fat. AR overexpression targeted by the col2.3 promoter was chosen for several reasons: the skeletal expression patterns for this promoter are both well-characterized and bone-selective (see 27, 28); the col2.3 promoter is not active in the periosteum but is strongly expressed in osteocytes and mineralizing nodules (29); the col2.3 promoter is also not active in osteoclasts; and androgens do not inhibit expression from the 2.3 kb promoter fragment (data not shown). Thus, the col2.3 promoter fragment directs expression of the fused transgene in bone, with strong expression still observed at the age of 3 months and even in animals as old as 6 months (29). Reports describing characterization of expression indicated strong expression in cells at osteogenic fronts of parietal bones, but the suture area was negative. In long bones, strong transgene expression was observed in most osteoblasts on endosteal surfaces, and in a large proportion of osteocytes in femurs throughout cortical bone, with no expression seen in periosteal fibroblasts (30). In the trabecular area of metaphyseal bone, strong expression was observed at all developmental stages (29).

Final comparison between AR2.3-transgenic and AR3.6-transgenic models to identify target cells in bone: One significant goal for this project was identification of target cells in the skeleton that mediate responses to androgen. Thus, it is instructive to compare the skeletal phenotypes that develop in the two distinct lines that we have generated, the AR2.3-transgenic mice described in these studies and the previously characterized AR3.6-transgenic model (13). In broad terms, the skeletal phenotype characterized in AR2.3-transgenic mice mirrors that described previously for AR3.6-transgenic males, indicating the specificity and reproducibility of the phenotypic consequences of bone-targeted androgen signaling. In common between the two models, we have shown increased trabecular bone volume, reduced formation at endosteal surfaces, reduced bone turnover and compromised biomechanical strength in male transgenic mice. With the exception of enhanced periosteal activity in AR3.6-transgenic males as noted below, neither model exhibits anabolic responses in the cortical bone compartment and instead both show inhibition of bone formation at the endosteal surface and compromised biomechanical properties. By comparing and contrasting the two AR-transgenic models, we propose that the commonalities in the bone phenotype between AR2.3-transgenic and AR3.6-transgenic mice arise from AR overexpression in mature osteoblasts and osteocytes, since both promoters are active in these cells. Thus, the increased trabecular bone volume, reduced bone turnover, reduced formation and decreased osteoblast vigor at endosteal surfaces, and compromised biomechanical strength with increased bone fragility observed in both models, are likely to be mediated at least in part by enhanced androgen signaling in mature osteoblasts/osteocytes.

Thus, androgen action in the mature mineralizing osteoblast results in reduced bone biomechanics and dramatically impaired matrix quality, with envelope-specific positive effects on bone formation only at periosteal surfaces. Compared with AR3.6-transgene overexpression throughout the osteoblast lineage (including bone marrow stromal cells, throughout osteoblast

differentiation including osteocytes), AR2.3-transgenic males do not show reduction in femur length and demonstrate much less inhibition of whole bone strength properties measured (stiffness, maximum load and work). However, AR2.3-transgenic mice show similar changes indicative of a low turnover phenotype, and similar increases in brittleness (i.e., decreased post-yield deflection), suggesting an analogous change in matrix quality and/or mineralization in both AR3.6-transgenic and AR2.3-transgenic mice. Thus, one **primary finding in *both* AR-transgenic lines is that androgen signaling in the osteoblast results in inhibition of mineral apposition rate (MAR) indices, strongly suggesting that direct androgen signaling in bone results in reduced osteoblast vigor and, as a consequence, poor matrix quality**.

The most striking contrast between the two AR-transgenic models we developed is observed at periosteal surfaces in AR3.6-transgenic males, which show increased cortical bone formation in the periosteum and dramatic intramembranous calvarial thickening. This finding was expected, given col3.6 transgene targeting to the periosteum and, conversely, the lack of expression at the same compartment with col2.3 transgene expression. The specificity of the periosteal anabolic effect in AR3.6-transgenic males is consistent with previous reports documenting the importance of androgen signaling in periosteal expansion (31). Thus, we propose that androgen inhibition of medullary bone formation at the endosteal surface in males may subserve an important physiological adaptive function, being key for appropriate spatial distribution and maintenance of the total amount/weight of bone in the cortical envelope. Combined, our data is consistent with the **hypothesis that androgens strongly promote the addition of cortical width through periosteal apposition, but balance that growth with inhibition of bone formation in the marrow cavity so that the skeleton does not become too heavy** (Appendix 6, 24). Thus, androgen-mediated **inhibition of bone formation** is a major consequence of AR signaling in the skeleton (Appendix 7, 25).

Model for androgen action in the skeleton mediated by AR transactivation. Androgen activation of AR influences a variety of target organs and skeletal sites, including marrow stromal cells, and trabecular, cortical and intramembranous bone compartments. Arrows indicate the changes associated with androgen action. In trabecular bone, androgen action preserves or increases trabecular number, has little effect on trabecular thickness, and thus reduces trabecular spacing. In cortical bone, AR activation results in reduced bone formation at the endosteal surface but stimulation of bone formation at the periosteal surface; correspondingly decreased periosteal but increased endosteal resorption results in no change in cortical area. In the transgenic model, AR activation in mature bone cells *in vivo* results in a low turnover phenotype, with inhibition of bone formation and inhibition of gene expression in both osteoblasts and osteoclasts. In the absence of compensatory changes at the periosteal surface, these changes are detrimental to overall matrix quality, biomechanics and whole bone strength.

Based on our characterization of AR-transgenic mouse models and other published reports, we **have developed the model presented above** (Appendix 2, 32) for the consequences of androgen signaling where the **effects of AR activation are distinct in different skeletal compartments** and can be characterized as envelope-specific (model shown below). Thus, in trabecular bone, androgens reduce bone turnover but increase trabecular volume through an

increase in trabecular number. In cortical bone, androgens inhibit osteogenesis at endosteal surfaces but increase bone formation at periosteal sites (Appendix 1, 13), to maintain cortical thickness yet displace bone further away from the neutral axis in males. Androgens also **positively influence bone at intramembranous sites** (Appendix 1, 13), likely in tissues with an embryonic origin derived from neural crest (see new data, Fig. 44). In addition, androgen administration increases muscle mass, partially mediated by effects on mesenchymal stem cell lineage commitment (33), likely to indirectly influence bone density through biomechanical linkage.   Forced exercise in studies employing AR3.6-transgenic mice would help test this hypothesis. To summarize our findings for this final report, complex skeletal analysis using morphological characterization by μCT, dynamic and static histomorphometric analysis, DXA, biomechanical testing and gene expression studies all **indicate that androgen signaling in mature osteoblasts inhibits osteogenesis at endosteal surfaces and produces a low turnover state** (Appendix 6, 24); these changes are detrimental to overall matrix quality, biomechanical competence, bone fragility and whole bone strength. It is possible that the observed inhibition of osteogenesis and lack of anabolic response, as a consequence of enhanced androgen signaling in mature bone cells, underscores an important physiological function for androgen in the skeleton: to maintain an appropriate spatial distribution of bone in the cortical envelope. The **strong inhibition of bone formation at the endosteal surface and increase in bone fragility may also underlie the limited therapeutic benefits observed with androgen therapy** (Appendix 5, 34). Our studies strongly suggest that because of the detrimental consequences of direct androgen signaling in bone we have documented, anabolic steroid abuse or high dose androgen therapy during growth and in healthy eugonadal adults is potentially damaging (Appendix 2, 32).

It should be noted that some of the negative consequences of AR overexpression in mature osteoblasts we have observed *in vivo* are consistent with our in vitro analyses. For example, there are reports, some in clonal osteoblastic cell lines, of effects of gonadal androgen treatment on differentiation, matrix production and mineral accumulation mediated by AR signaling (35-37). These findings are variable however, with other reports of no effect or even inhibition of osteoblast markers (38-40), consistent with our gene expression analysis in AR-transgenic mice. In addition, the effect of androgens on osteoblast proliferation is controversial. We have previously demonstrated that either stimulation or inhibition of osteoblast viability by androgen can be observed, and these effects are dependent on the length of treatment. Transient administration of nonaromatizable DHT can enhance transcription factor activation and osteoblast proliferation, while chronic treatment inhibits both mitogenic signaling and MAP kinase activity (Appendix 1, 41). Chronic DHT treatment *in vitro* also results in enhanced osteoblast apoptosis (Appendix 3, 42). Thus, during this grant period we have shown that chronic **DHT treatment inhibits osteoblast proliferation, enhances osteoblast apoptosis, and suppresses osteoblast differentiation and mineralization** (Appendix 4, 40). New data presented in this final progress report documents the **direct cell-autonomous inhibition of osteoblast proliferation, matrix maturation and mineralization by androgen signaling**. Inhibition is strongest in cells that have the overexpression of AR throughout osteoblast differentiation in AR3.6-tg mice compared with overexpression limited to mature cells in AR2.3-tg mice (Appendix 7, 25). Thus, our *in vitro* analyses are consistent with the **detrimental changes in matrix quality and dramatic inhibition of osteoblast vigor** we have quantified in both AR-transgenic models *in vivo*.

Thus, characterization of the consequences of bone-targeted overexpression revealed a skeletal phenotype in male transgenic mice versus littermate controls, with little difference between the females. Collectively, the phenotype observed in male transgenic mice is likely dependent on the higher levels of androgen (~10-fold) circulating in males vs. females. In our studies, we have found that AR overexpression in the mature osteoblast population *in vivo*

results in a low turnover state with increased trabecular bone volume, but a significant reduction in cortical bone area due to inhibition of bone formation at the endosteal surface and a lack of marrow infilling. Every measure of biomechanical responsiveness is significantly inhibited. The most remarkable aspect of the phenotype is a dramatic reduction in osteoblast vigor. *Ex vivo* analysis of osteoblast differentiation in primary culture demonstrates that androgen directly inhibits proliferation, osteoblast differentiation, mineral accumulation and mineralized nodule formation. Data are also consistent with an inhibition of osteoclastogenesis, consistent with the low turnover phenotype. Thus, primary outcomes of analyses demonstrate that enhanced androgen signaling in mature osteoblasts/osteocytes inhibits cortical bone formation, and results in changes that are detrimental to matrix quality, biomechanical competence and whole bone strength. The detrimental effects of androgen on bone were observed in both AR-transgenic lines. **This outcome was not predicted based on the literature available when these studies were initiated, and raise concerns regarding androgen administration during development or in eugonadal adults**.

Reductions in osteoblast vigor present *in vivo* likely reflect inhibition of osteoblast proliferation, differentiation and mineralization observed *in vitro* in primary cultures and also in the signaling pathways identified in gene profiling analysis from whole bone by bioinformatic strategies. The striking inhibition of osteoblast proliferation and differentiated function observed in primary cultures suggests that **inhibitory androgen action is cell-autonomous**, with direct negative regulation in osteoblasts. Since osteocytes are the most abundant cell type in bone, and AR levels are highest in osteocytes, these cells are likely a primary target for negative androgen signaling in bone.

Gene expression profiling analysis suggests that osteocytes respond to chronic DHT treatment by modulation of signaling pathways that highlight the importance of TGFβ/BMP/Activin signaling, apoptosis/cell death, and modulation of signal transduction through membrane receptors, particularly Wnt pathways. The identification of important pathways may **provide insight into the development of therapeutics for treatment of low bone mass diseases, particularly to minimize inhibitory effects of direct androgen signaling on bone cells**.

Finally, results suggest that **AR signaling in neural crest-derived tissue results in enhanced osteoblastogenesis and bone matrix production, while AR signaling in MSC results in inhibition of the osteoblast lineage but changes in body composition (increased lean mass and decreased fat mass in both sexes) that would be beneficial in the aging population**. Since androgen increases osteoblastogenesis at the periosteal surface, we **hypothesize that periosteal osteoblasts are thus derived from a neural crest stem cell origin**. It may therefore be advantageous to focus future development of bone anabolic therapies in neural crest stem cell lineages.

Key Research Accomplishments
Accomplishments for the entire research program (2004-2009) directly characterizing androgen action in the skeleton:

Peer reviewed publications:

- **Wiren KM**, Zhang X-W, Toombs AR, Gentile MA, Kasparcova V, Harada S-I, Jepsen KJ: Targeted overexpression of androgen receptor in osteoblasts: unexpected complex bone phenotype in growing animals. Endocrinology 145:3507-3522, 2004

- **Wiren KM**: Androgen action and bone growth: it's location, location, location. Curr Opin Pharmacol 5:626-632*, 2005*
- ***Wiren KM**, Toombs AR, Semirale AA, Zhang X-W. Apoptosis associated with androgen action in bone: requirement of increased Bax/Bcl-2 ratio. Bone 38:637–651, 2006
  *Selected for journal cover
- Bi LX, **Wiren KM**, Zhang X-W, Oliveira GV, Klein GL, Mainous EG, Herndon DN: Direct effect of oxandrolone treatment on human osteoblastic cells is modest. J Burns Wounds 6:53-64, 2007
- Semirale A. A., **Wiren KM**: Androgen administration has therapeutic advantages in the hypogonadal, but should be approached with caution in healthy adults. J Musculoskelet Neuronal Interact 2007; 7(4):361-367
- **Wiren KM**, Semirale A. A., Zhang X-W, Woo A, Tommasini SM, Price C, Schaffler M, Jepsen KJ: Targeting of androgen receptor in bone reveals a lack of androgen anabolic action and inhibition of osteogenesis. A model for compartment-specific androgen action in the skeleton. Bone 43:440–451, 2008
- **Wiren KM**, Semirale AA, Hashimoto JG, Zhang X-W: Signaling pathways implicated in androgen regulation of endocortical bone. Bone doi:10.1016/j.bone.2009.10.039, 2009

Chapters:

- **Wiren KM**: Androgens and Skeletal Biology: Basic Mechanisms. In: Osteoporosis, Third Edition (R Marcus, Feldman D, Nelson D, Rosen C, Eds), Academic Press, San Diego, CA, pp. 425-449*, 2008*
- **Wiren KM**: Androgens: receptor expression and steroid action in bone. In: Principles of Bone Biology, Third edition (JP Bilezikian, LG Raisz and TJ Martin, Eds), Academic Press, San Diego, CA, pp 1001-1023, 2008
- **Wiren KM**: Androgen Action in Bone: Basic Cellular and Molecular Aspects. In: Osteoporosis: Pathophysiology and Clinical Management, Second Edition (R Adler, Ed), The Humana Press, Inc., Totowa, NJ, pp. 357-381*, 2009*
- Orwoll ES, **Wiren KM**: Androgens and bone: basic aspects. In: Osteoporosis in men: the effects of gender on skeletal health, Second Edition (ES Orwoll, JP Bilezikian and D Vanderschueren, Eds), Academic Press, San Diego, CA, pp. 295-317, 2009
- **Wiren KM**: Androgens and skeletal biology: basic mechanisms. In: Fundamentals of Osteoporosis (R Marcus, Ed), Elsevier, San Diego, CA, *in press*, 2009

Abstracts:

- **Wiren KM**, Toombs A, Zhang X-W: Enhanced apoptosis associated with anabolic actions of androgens in AR-transgenic mice: requirement of reduced bcl-2/bax ratio. (Abstract #M226) J Bone Miner Res, 2004
- ***Wiren KM**, Toombs A, Kasparcova V, Harada S, Zhang X-W: Androgens inhibit osteoclast activity in AR-transgenic mice through reductions in RANKL/OPG ratio. (Abstract #F202/SA202) J Bone Miner Res, 2004
  *Selected for the Plenary Poster Session award
- ***Wiren KM**, Toombs AS, Matsumoto AM, Zhang X-W: Androgen receptor transgenic mice have decreased body fat, reduced adipogenesis and an increased osteoblast differentiation program. (Abstract #1198) J Bone Miner Res, 2005
  *Selected for Oral Presentation
- **Wiren KM**: Direct androgen action in the skeleton. (Wellness/Fitness Research Abstract #121) PRMRP Military Health Research Forum, 2006

- Semirale A, Zhang X-W, **Wiren KM**: Androgen receptor overexpression in precursor cells and not mature osteoblasts influences body composition by reducing adiposity and increasing lean mass. (Abstract#SU466) J Bone Miner Res, 2006
- ***Wiren KM**, Zhang X-W, Semirale A, Price C, Woo A, Jepsen KJ: Targeted androgen action in mature vs. preosteoblasts eliminates periosteal stimulation and reduces bone quality during growth. (Abstract#1015) J Bone Miner Res, 2006
     *Selected for Oral Presentation ASBMR
- Zhang X-W, Semirale A, **Wiren KM**: Dissection of Androgen Receptor Signaling: Reconsideration of Direct Anabolic Action in Mature Bone. (Abstract#T202) J Bone Miner Res, 2007
- Semirale A, Zhang X-W, **Wiren KM**: DHT Treatment Reverses Gonadectomy-Induced Changes in Fat and Lean Mass in Male but Not Female Mice. (Abstract#T203) J Bone Miner Res, 2007
- Semirale A, **Wiren KM**: Therapeutic Considerations Regarding Androgen Administration. Sun Valley Workshop on Skeletal Tissue Biology, 2007
     *Awarded Alice L. Jee Memorial Young Investigator Award
- Zhang X-W, **Wiren KM**: Overexpression of androgen receptor in mature osteoblasts and osteocytes inhibits osteoblast differentiation. (Abstract #SU192) J Bone Miner Res, 2008
- Semirale A. A., **Wiren KM**: DHT Administration Is Effective for the Prevention of Hypogonadal Bone Loss. (Abstract #SU193) J Bone Miner Res, 2008
- Ayala P, Uchida M, **Wiren KM**, Hurn P: Androgens exert neuroprotection on targeted overexpression of androgen receptor in mice brain from ischemia and in PC12 from oxidative stress and apoptosis. (Abstract 556.22/CC9) Society for Neuroscience, 2008

Reportable Outcomes:

   Seven peer-reviewed publications, five book chapters and twelve abstracts were published over the course of this grant from 2004-2009, characterizing androgen action in the skeleton. This year, one peer-reviewed publication was published (with several more in preparation). In addition, substantial progress was seen in the new subcontract that was established last year for μCT analysis for AR2.3- and AR3.6-transgenic mice. We have completed surgeries and androgen replacement in two distinct hypogonadal models in adult animals; one being a therapeutic strategy (low turnover) to improve bone mass and the other being a preventative approach (high turnover) to inhibit losses. DXA analysis has been completed and significant progress has been made with μCT analysis. Additional publications will be submitted when μCT analysis is completed. Current results suggest that androgen treatment is not anabolic to increase bone mass, but instead results in an anti-resorptive response that preserves bone. Inhibitory responses in endocortical bone may be mediated by alteration of the TGFβ superfamily, especially through inhibition of BMP signaling. AR action inhibits MSC numbers and reduces osteoblastogenesis in MSC cultures.

   Based on our extensive analysis, Dr. Wiren is viewed as an expert in the consequences of androgen signaling in the skeleton. Thus, she was invited to present her work at Osteomen 2008 in Santa Margherita, Italy and to participate in the 2009 Musculoskeletal Global Therapeutic Expert Forum (Selective Androgen Receptor Modulators--Discussion Group 2) by Merck & Co in Jersey City, NJ.

   Future directions for the characterization of androgen action *in vivo* includes analysis of AR signaling in neural crest stem cells vs. MSC. Information derived from such analysis could identify a novel anabolic strategy to build bone, with androgen targeted to neural crest stem

cells rather than MSC. We also believe it would be worthwhile to determine possible extension of longevity as a consequence of alterations in body composition and brain function. These studies would also entail confirmation of increased muscle strength/function as a consequence of AR3.6-transgene expression in MSCs.

Personnel:
Kristine Wiren, PI and personnel with Dr. Wiren, including Dr. Anthony Semirale, Dr. Xiao-Wei Zhang, Joel Hashimoto and Amber Toombs
Subcontract personnel: Urszula Iwaneic, Russell Turner, Karl Jepsen

Conclusions:
These results have provided new insight into the importance of androgen action, through distinct AR transactivation, in mediating bone quality and changes that underlie envelope-specific responses. Our investigations of the mouse skeleton reveal that androgen signaling in immature osteoblasts and periosteal cells increases bone formation at the periosteal envelope (particularly in calvaria) and also influences processes that determine body composition and whole bone strength in AR3.6-transgenic mice. In contrast, analysis from AR2.3-transgenic mice suggests that signaling in mature osteoblasts/osteocytes primarily mediates the effects of androgens on matrix quality and/or mineralization with a negative impact on osteoblast vigor, and at least partially influences the effects of androgens to reduce bone turnover. A similar relationship may also exist in the human skeleton.

Thus, the effects of androgens are pervasive, influencing many organ systems in the body including bone. However, the mechanisms through which AR signaling influence osteoblast function remain controversial. We have shown previously that in mature osteoblasts, androgens inhibit osteogenesis and that AR signaling in vivo inhibits bone formation by differentiated osteoblasts and has detrimental consequences on matrix quality, bone fragility and whole bone strength (13, 24). We have also shown chronic DHT treatment inhibits both mitogenic signaling and MAP kinase activity in osteoblasts (41), and enhances osteoblast apoptosis (42). In order to evaluate cell autonomous affects of androgen on osteoblast function and identify important signal transduction pathways that are affected, mice with targeted AR overexpression were used as a source of primary osteoblasts. We show that androgen directly inhibits normal osteoblast proliferation, matrix production and mineralization. Overexpression of AR exacerbates the detrimental effects of DHT treatment. Consistent with the osteocyte as a target of androgen signaling, addition of DHT during the late stages of differentiation results in the largest decrease in marker protein levels. The results indicated that AR overexpression results in cell-autonomous inhibitory effects of androgen on proliferation and mineralization in vitro. These results raise concerns that with increasing androgen action (as for example associated with anabolic steroid abuse) in young animals with still-growing skeletons, the bone matrix material is likely to be more stiff, less ductile and more damageable, and suggest that young anabolic steroid abusers may be at higher risk of stress fractures. Importantly, supplemental levels of androgen in eugonadal adults are also detrimental. In contrast, analyses in the mature adult indicate that androgen may be useful to treat reductions in bone mineral in hypogonadal males.

In addition, androgen administration increases muscle mass, partially mediated by effects on stem cell lineage commitment (33), likely to indirectly influence bone density through biomechanical linkage. But as we have shown, improvement body composition after androgen replacement is observed only in hypogonadal males. At the same concentration and for the

same duration, hypogonadal females do not demonstrate the same improvement with androgen replacement. Supplemental androgen in eugonadal adults tends to worsen, not improve, body compositional changes that occur with aging.

Some of the negative consequences of AR overexpression in mature osteoblasts we have observed in vivo are consistent with our *in vitro* analyses. For example, there are reports, some in clonal osteoblastic cell lines, of effects of gonadal androgen treatment on differentiation, matrix production and mineral accumulation mediated by AR signaling (35-37). These findings are variable however, with other reports of no effect or even inhibition of osteoblast markers (38-40), consistent with our gene expression analysis in AR-transgenic mice. In addition, the effect of androgens on osteoblast proliferation is controversial. We have previously demonstrated that either stimulation or inhibition of osteoblast viability by androgen can be observed, and these effects are dependent on the length of treatment. Transient administration of nonaromatizable DHT can enhance transcription factor activation and osteoblast proliferation, while chronic treatment inhibits both mitogenic signaling and MAP kinase activity (41). Chronic DHT treatment *in vitro* also results in enhanced osteoblast apoptosis (42). Thus, these in vitro reports are consistent with the detrimental changes in matrix quality and osteoblast vigor we observe in the AR-transgenic model *in vivo*.

In summary, one of the consequences of enhanced androgen signaling is **altered whole bone quality and susceptibility to damage in the young, which may be revealed under extreme physical conditioning such as that experienced during military training**. Our data also indicate a concern with androgen supplementation in eugonadal adults, both male and female. Finally, it does appear that androgen may be **effective in improving body composition in hypogonadal males and females, including increased lean and reduced fat mass**. While AR3.6-tg mice are more responsive than wild-type, AR2.3-tg mice appear to have reduced sensitivity to the androgen-mediated improvement in body composition. Combined, these results support the central hypothesis of distinct androgen signaling throughout osteoblast differentiation and **identifies potential therapeutic targets for age-related fragility**.

Androgen action in the skeleton is complex and will likely not provide for dramatically improved skeletal dynamics in the still-growing skeleton or the eugonadal adult, although the effect in the hypogonadal male is more positive. As noted, **this outcome was not predicted based on the literature available when these studies were initiated, and raise concerns regarding androgen administration during development or in eugonadal adults**. It may be that the identification of important pathways can provide insight into the development of therapeutics for treatment of low bone mass diseases, particularly to minimize inhibitory effects of direct androgen signaling on bone cells on BMP signaling. Interestingly, pathways that were modified by androgen signaling in the periosteal compartment were focused on stem cells. Following up on this observation, it appears that **osteoblastogenesis is positively affected by androgen in the osteoblast lineage derived from *neural crest* rather than mesodermal mesenchymal stem cells**. Enhanced androgen signaling in neural crest derivatives thus has the potential to provide an improved therapeutic target for an anabolic skeletal response at the periosteal surface. Since increasing bone width provides for greatly enhanced biomechanical strength, such an approach may be beneficial under conditions of skeletal strain.

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Targeted Overexpression of Androgen Receptor in Osteoblasts: Unexpected Complex Bone Phenotype in Growing Animals

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The androgen receptor (AR), as a classic steroid receptor, generally mediates biologic responses to androgens. In bone tissue, both AR and the estrogen receptor (ER) are expressed in a variety of cell types. Because androgens can be converted into estrogen via aromatase activity, the specific role of the AR in maintenance of skeletal homoeostasis remains controversial. The goal of this study was to use skeletally targeted overexpression of AR as a means of elucidating the specific role(s) for AR transactivation in bone homeostasis. Rat AR cDNA was cloned downstream of a 3.6-kb $\alpha1(I)$-collagen promoter fragment and used to create AR-transgenic mice. AR-transgenic males gain less weight and body and femur length is shorter than wild-type controls, whereas females are not different. AR-transgenic males also demonstrate thickened calvaria and increased periosteal but reduced endosteal labeling by fluorescent labeling and reduced osteocalcin levels. High-resolution micro-computed tomography shows normal mineral content in both male and female AR-transgenic mice,

but male AR-transgenics reveal a reduction in cortical area and moment of inertia. Male AR-transgenics also demonstrate an altered trabecular morphology with bulging at the metaphysis. Histomorphometric analysis of trabecular bone parameters confirmed the increased bone volume comprised of more trabeculae that are closer together but not thicker. Biomechanical analysis of the skeletal phenotype demonstrate reduced stiffness, maximum load, post-yield deflection, and work-to-failure in male AR-transgenic mice. Steady-state levels of selected osteoblastic and osteoclastic genes are reduced in tibia from both male and female transgenics, with the exception of increased osteoprotegerin expression in male AR-transgenic mice. These results indicate that AR action is important in the development of a sexually dimorphic skeleton and argue for a direct role for androgen transactivation of AR in osteoblasts in modulating skeletal development and homeostasis. (*Endocrinology* 145: 3507–3522, 2004)

T HE MOLECULAR PATHWAYS controlling bone formation in either normal individuals or in pathophysiologic disease states are not well understood. This question is of significant importance because osteoporosis, a low bone mass disease associated with an increased risk of fracture, is the most prevalent degenerative disease in developed countries (1). Osteoporosis is generally characterized by a relative decrease in bone formation (mediated by osteoblasts) *vs.* bone resorption (mediated by osteoclasts) and is often coupled with a hypogonadal state in both men (2) and women. Although both estrogen and androgen circulate in both genders, the influence of estrogen and androgen on the skeleton is distinct as shown by divergent responses to gonadectomy in either gender (3), particularly with respect to bone size and

periosteal apposition. Furthermore, combination therapy with estrogen and androgen in postmenopausal women is more beneficial than either steroid alone (4–6), indicating nonparallel pathways of action. Estrogens are thought to act to maintain adult bone mass predominantly through an inhibition of bone resorption by the osteoclast, *i.e.* as antiresorptive agents, which protect the skeleton from further loss of bone. Nonaromatizable androgens such as 5α-dihydrotestosterone (DHT), on the other hand, are characterized as anabolic agents that increase bone mass by stimulation of bone formation (7, 8), and thus represent an important therapeutic class that may have the potential to rebuild lost bone. However, an understanding of the pathways influenced by androgen in the osteoblast is currently very limited.

In general terms, the skeletal response to systemic androgen therapy has been characterized as increased trabecular and cortical bone mass, increased bone width with surface periosteal expansion and a lack of inner endosteal deposition. This is observed in the setting of inhibition of resorption due to reduced osteoclast activity, that may not be as significant as that seen with estrogen replacement (see Ref. 9). There has been speculation that the positive effects of androgens on the skeleton may be mediated indirectly through increased muscle mass in biomechanical linkage, thought to have beneficial effects on bone density. However, analysis of

the myostatin null mouse, the so called "Mighty mouse," with dramatic hypertrophy of muscle that shows no difference in bone at the cortical midshaft, suggests this may not entirely be the case (10). In addition, fat mass, not lean mass, is better associated with improved bone mineral density (11). It has also been proposed that sex steroids can act nonspecifically through nongenomic actions at either ER or AR (12, 13), although recent data suggest that genomic signaling may be the more significant regulator *in vivo* (14, 15).

Analysis of AR signaling *in vivo* has been approached genetically both with global receptor knockouts (16, 17) and with the testicular feminization model (18). The global loss of AR results in high-turnover osteopenia and reduced trabecular bone volume, with a significant stimulatory effect on osteoclast function. Experimental strategies such as surgical or pharmacological intervention have also been employed to characterize androgen signaling. Distinct effects of androgen are seen with gonadectomy when comparing the effects of orchidectomy in male (ORX) *vs.* ovariectomy in the female (OVX) rats. OVX in the female results in decreased trabecular area in the metaphysis with increased osteoclast number and an increase in serum calcium, but in cortical bone at the diaphysis, an increase at the periosteal surface with circumferential enlargement but a decrease in endosteal labeling (3). These results suggest that estrogen protects trabecular bone predominantly through inhibition of osteoclast activity/recruitment, and exhibits an inhibitory action at the periosteal surface. In the male, ORX also resulted in decreased trabecular area in the metaphysis with increased osteoclast number, resulting in trabecular osteopenia in the secondary spongiosa (19). In contrast with the female, cortical bone periosteal formation was reduced. Replacement with nonaromatizable DHT pellets for 3 wk in the male prevented the loss of trabecular bone (19). In the intact animal, the stimulation of endosteal formation by estrogen compensates for the lack of periosteal formation, thus leading to no difference in biomechanical strength (20).

Although these approaches have advanced our understanding of steroid action in bone, they have limitations. For example, surgical removal of gonadal tissue (*i.e.* castration) to eliminate hormone production or pharmacological treatment with a receptor antagonist such as hydroxyflutamide may affect multiple organ systems and result in secondary effects. Global knockout of AR necessarily results in loss of AR function in all tissues, which can have developmental consequences and indirect effects on bone balance. Additional confusion regarding the specific action of androgens on the skeleton results from the fact that testosterone can be metabolized to estrogen via aromatase activity. Thus, some androgen action may result from ER-dependent activation after conversion to 17β-estradiol. For these reasons, creation of transgenic lines with bone-targeted overexpression of AR should enhance our understanding of the specific role for androgen through AR transactivation in skeletal tissue. Our data show that AR overexpression in the osteoblast lineage has dramatic effect on bone quality and trabecular morphology in male AR-transgenic mice, and argue for a direct role for androgen transactivation of AR in osteoblasts in modulating skeletal development and homeostasis.

Materials and Methods

Cloning of expression plasmids

The pBR327-based plasmid col3.6-βgal-ClaPa containing the rat type I α_1 collagen (ColIα1) promoter sequence −3518 to +115, served as the starting vector (generously provided by Dr. David Rowe, University of Connecticut Health Center, Farmington, CT). The *Bam*HI site at −3145 in col3.6-βgal-ClaPa was removed by introducing a point mutation using *in vitro* mutagenesis to create the modified col3.6E-βgal-ClaPa. Briefly, a 50-μl thermal cycling elongation reaction consisted of 50 ng col3.6-βgal-ClaPa plasmid, 0.4 μM primers each, 200 μM deoxynucleotide triphosphate, and 2.5 U *Pfu*Turbo DNA high-fidelity polymerase. The primers used contain a point mutation (*bold/italicized letters*) that destroys the *Bam*HI site (forward: 5'-CACCACACACCTAGGA*C-C*CACCCACAGATTTTGC-3' and reverse: 5'-GCAAAATCTGTGGGT-GG*G*TCCTAGGTGTGTGGGGTG-3'). The reaction was 94 C for 45 sec, 55 C for 45 sec, and 68 C for 20 min for 20 cycles. The combination of low cycle number and high-fidelity polymerase minimizes unwanted mutations. After the reaction, 10 U *Dpn*I was added for 1 h at 37 C. *Dpn*I is specific for methylated and hemimethylated DNA, and selectively digests the parental and hybrid plasmid DNAs. Positive clones were identified by the *Bam*HI restriction pattern. To add *Bam*HI sites to the rat AR cDNA (provided by Dr. Shutsung Liao, University of Chicago, Chicago, IL), PCR primers were designed with *Bam*HI ends as follows: forward 5'-*GGATC*CATGGAGGTGCAGTTAGGGCT-3', reverse 5'-*GGATC*CTCACTGTGTGGAAATAGA-3'. PCR conditions were 94 C for 30 sec, 55 C for 30 sec, and 68 C for 3 min for 30 cycles with 2.6 U Expand High Fidelity PCR Systemenzyme mix (Roche Applied Science, Indianapolis, IN) in 50 μl reaction. The PCR product was T/A cloned in pCR 2.1-TOPO vector (Invitrogen Life Technologies, Carlsbad, CA). The *Bam*HI-rAR was released by digestion with *Bam*HI. Finally the *Bam*HI-rAR fragment was cloned into the *Bam*HI site in the modified col3.6E-βgal-ClaPa (after removal of the βgal cDNA sequences), to give the expression construct termed col3.6-AR (hereafter referred to as colAR) shown in Fig. 1A. The correct sequence and orientation of the AR insert was verified by direct DNA sequencing.

Generation of Col3.6-AR transgenic mice

The colAR expression plasmid was digested with *Cla*I to release the colAR transgene, and the transgene DNA fragment was isolated by agarose gel electrophoresis and purified by electroelution. The colAR transgene (2 μg/ml) was microinjected in to the male pronucleus of fertilized one-cell mouse B6D2F2 embryos by the Oregon Health & Science University (OHSU) Transgenic Mouse Facility. F1 embryos were obtained from matings of C57BL/6 males × DBA/2J females (B6D2F1). The injected embryos were reimplanted into pseudopregnant B6D2F1 female mice. Founder mice were identified by PCR genotyping and mated with B6D2F1 (The Jackson Laboratory, Bar Harbor, ME) to produce F1 litters. The generation and use of transgenic mice were performed according to institutional, local, state, federal, and National Institutes of Health guidelines for the use of animals in research under an Institutional Animal Use and Care Committee-approved protocol.

DNA extraction, Southern analysis, and PCR genotyping

Genomic DNA was purified from a small piece of tail tissue obtained at the time of weaning using a standard proteinase K and phenol-chloroform extraction procedure. Genotyping was performed by PCR analysis using colAR-GT primers forward 5'-TAGCACCTCTGGC-CCATGTA-3' and reverse 5'-TCCTGCCGCTGCTGTAAACA-3'. These primers were designed to specifically amplify the transgene by including part of the rat collagen sequence in the ClaPa constructs and part of the AR sequence as shown in Fig. 1A. Primers were derived using OLIGO Software from Molecular Biology Insights, Inc. (Cascade, CO) and purchased from Fisher Scientific (Pittsburgh, PA). A single insertion site for the transgene was confirmed by Southern blot using the PCR fragment as a probe. Copy number was determined by real-time PCR analysis using genomic DNA employing colAR-RT primers described in real-time RT-PCR analysis and primers for the endogenous mouse AR gene forward 5'-GGAATTCGGTGGAAGCTACA-3' and reverse 5'-CCGGGAGGTGCTATGT-3'. Eight mice were found to be positive for the presence of the transgene, and founders of both genders were mated

FIG. 1. Generation of transgenic mice with bone-targeted AR overexpression. A, A schematic representation of the col3.6 AR transgene. B, Southern analysis for characterization of transgenic animals. Genomic DNA was isolated from both AR-transgenic founder lines (104 and 106), digested with *Eco*RV, *Pst*I, or *Sal*I and subjected to Southern blot analysis after hybridization with the colAR transgene product. Analysis indicates a single insertion site. C, Copy number of the AR transgene (tg-AR) relative to the endogenous AR (endog-AR) gene was estimated by real-time PCR analysis of genomic DNA (~5–7 for both lines). bGH, Bovine GH; PA, polyadenylation signal.

to B6D2F1 mice to establish transgenic lines. Results are presented using two independent AR-transgenic lines (104 and 106) with moderate copy number and single integration site.

Animals

AR-transgenic mice were bred to B6D2F1 mice (The Jackson Laboratory); both genders were employed. The litters were housed with the dam until weaning at 21 ± 2 d of age, at which time they were housed three to four per cage in isosexual groups with mice of the same genotype. The mice had free access to tap water and were fed a diet containing 1.14% calcium, 0.8% phosphorous, 2200 IU/kg vitamin D3, 6.2% fat, and 25% protein (Purina PMI Nutrition International, St. Louis, MO). All animals were weighed weekly, and body length (nose to rump) was determined at weekly or monthly intervals, respectively, over 6 months (n = 4–5).

The animals were killed under CO_2 narcosis by decapitation. Before killing, 8-wk-old mice received two fluorochrome labels by ip injection for evaluation of bone dynamics [oxytetracycline hydrochloride (Sigma, St. Louis, MO) at 30 mg/kg 10 d before killing and calcein green (Sigma) at 10 mg/kg 3 d before killing]. The calvaria and femora were dissected and cleaned from surrounding tissue. The left femur was immersed in 4% paraformaldehyde fixative for 24 h at 4 C and subsequently kept in 100% ethanol until histomorphometric analysis. The right femur was used for measurement of cortical and trabecular volumetric density and geometry by micro-computed tomography (micro-CT) *ex vivo*, followed by destructive analysis of whole bone biomechanical properties described below. The length of the femur was measured from the femoral head to the distal condyles. In addition, calvaria, liver, kidney, tendon, ear, muscle, skin, heart, fat, and spleen were collected for RNA isolation or immunocytochemical analyses. For RNA isolation, tibia was cleaned of muscle tissue and aseptically dissected. After removal of the epiphyseal area, marrow is briefly flushed with sterile saline and the bone frozen in liquid nitrogen and stored at −80 C until RNA isolation as described below.

Real-time RT-PCR analysis

Analysis of colAR transgene expression in different tissues was performed with the iCycler IQ Real Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA) using a one-step procedure on deoxyribonuclease (DNase)-treated total RNA (see Table 1). RNA was isolated using the RNA Stat-60 kit (Tel-Test, Inc., Friendswood, TX). Contaminating DNA was removed by RQ1-DNase (Promega, Madison,

TABLE 1. Real-time RT-PCR analysis of col3.6AR transgene mRNA expression in tissues from AR-transgenic mice

Tissue	ColAR level	Fold difference
Calvaria	1.000 ± 0.325	N/A
Liver	0.008 ± 0.001	−125.0
Kidney	0.019 ± 0.001	−52.6
Tendon	0.014 ± 0.001	−71.4
Ear	0.002 ± 0.001	−500.0
Muscle	0.051 ± 0.007	−19.6
Skin	0.012 ± 0.003	−83.3
Heart	0.014 ± 0.001	−71.4
Fat	0.002 ± 0.001	−500.0
Spleen	0.012 ± 0.001	−83.3

Tissues listed were harvested from male AR-transgenic mice, and total RNA was isolated (n = 5). Expression of the colAR transgene was evaluated by real-time RT-PCR analysis after normalization to the value for 18S rRNA. Data are expressed relative to the expression level in calvaria as mean ± SEM. N/A, Not applicable.

WI) digestion and phenol-chloroform extraction or by Zymo-spin column purification following manufacturer's recommendations (Zymo Research, Orange, CA). Twenty nanograms of RNA were reverse transcribed and amplified in a 25-μl reaction mix containing 1× QuantiTect SYBR Green RT-PCR Master Mix (QIAGEN, Valencia, CA) and 0.5 μM each primer. ColAR-RT primers were 5'-GCATGAGCCGAAG-CTAAC-3' and 5'-GAACGCTCCTCGATAGGTCTTG-3' and specifically amplified colAR using sites in the collagen untranslated region and AR near to those used for genotyping (Fig. 1A). After PCR, reaction products were melted over the temperature range 55–95 C in 0.5 C increments, 10 sec per increment to ensure only the expected PCR product was amplified per reaction. The efficiency of amplification was determined for each primer set from serial dilutions and did not vary significantly from 2. Expression of osteoblast and osteoclast-specific genes was determined by real-time RT-PCR analysis using Applied Biosystems 7700 Sequence Detector System (Applied Biosystems, Foster City, CA). Total RNA was extracted from tibia diaphysis using Purescript RNA Isolation Kit (Gentra Systems, Minneapolis, MN) followed by DNase treatment on RNeasy Micro Columns (QIAGEN) according to manufacturer's instructions. cDNA was prepared from pooled RNA (n = 5) using TaqMan Reverse Transcription Reagents (Applied Biosystems). The PCR was performed from cDNA in duplicates using

TaqMan PCR Core Reagent Kit (Applied Biosystems) with 200 nM primers and a probe, and 10 μl of cDNA template. Gene-specific primers and fluorescence-labeled probes [5'-reporter dye: FAM (6-carboxyfluorescein), 3'-quencher dye: TAMRA (6-carboxymethyl rhodamine)] (Table 2) were designed using Primer Express (version 1.5) software (Applied Biosystems) and were synthesized by Applied Biosystems. Relative expression of the RT-PCR product was then determined using the comparative $\Delta\Delta C_t$ method (21). 18S rRNA was used to normalize expression in each sample. Fold regulation was then determined by normalizing all values to the mean of the relative expression for the control group.

Serum biochemistry

Serum specimens from 8-wk-old female and male mice of both genotypes were collected and stored at -20 C until analysis was performed (n = 2–7). Blood samples were obtained under anesthesia by cardiac puncture. Serum 17β-estradiol was measured by RIA using Immuchem Double Antibody 17β-Estradiol RIA (ICN Biomedicals Inc., Costa Mesa, CA) with a sensitivity of 7.2 pg/ml. Assays were performed using 50 μl serum aliquots in duplicate following manufacture's recommendations. Intraassay variation was 5%, and interassay variation was 9%. Testosterone was measured by enzyme linked immunoassay from Diagnostic Automation Inc. (Calabasas, CA), with a sensitivity of 0.05 ng/ml. Assays were performed using 10-μl serum aliquots in duplicate following manufacturer's recommendations. Serum osteocalcin, a marker of bone turnover, was quantitated by ELISA (Biomedical Technologies Inc., Stoughton, MA) in 5-μl aliquots with a sensitivity of 0.1 ng/ml. Intraassay variation was 6%, and interassay variation was 8%. Serum calcium was determined in duplicate in 25-μl samples using the complexone method (Raichem, San Diego, CA). Mouse serum osteoprotegerin (OPG) as determined by OPG immunoassay kit with sensitivity of 4.5 pg/ml (R&D Systems, Minneapolis, MN). Briefly, serum samples were 5-fold diluted and incubated with monoclonal antibody specific for mouse OPG for 2 h at room temperature. After washing for four times, samples were incubated with antimouse OPG conjugated to horseradish peroxidase for 2 h at room temperature. Substrate solution was added and incubated for 30 min. OD was determined at 450 nm. The intraassay and interassay CV are 5.5–7.9% and 6.9–7.4%.

Histochemical analysis of calvaria

Histochemical analysis was performed on representative calvaria from offspring of two independent founder lines (104 and 106). Calvaria were fixed in freshly prepared 4% paraformaldehyde in borate buffer for 48 h. After decalcification in Immunocal (Decal Corp., Tallman, NY) for 2–3 wk, sections were processed by dehydration, paraffin infiltration, and embedding (melting point: 58–62 C). Tissue sections (5–6 μm) were cut with a microtome and floated onto positively charged slides (Superfrost, Fisher Scientific). Sections were deparaffinized and hydrated through a xylene and graded ethanol series. For hematoxylin and eosin (H&E) staining, the sections were placed in hematoxylin for 5 min, in 1% acid alcohol for a few seconds, and in eosin for 5 min.

For AR immunocytochemistry, slides were placed in freshly prepared 3% H_2O_2 in methanol for 10 min to inhibit endogenous peroxidase activity. A high-temperature antigen unmasking technique was performed by immersing slides in boiling 0.01 M citrate buffer (pH 6.0) for 15 min, then slides were subjected to immunohistochemical staining. Polyclonal rabbit AR antibody (PA1-111A) was purchased from Affinity

Bioreagents Inc. (Golden, CO) and used at 4 μg/ml. The PA1-111A AR antibody maps to the N terminus of the receptor and does not recognize other members of the steroid receptor family. Controls for nonspecific binding were incubated with rabbit nonimmune IgG. The sections were incubated with antibody overnight at 4 C. Secondary biotinylated antirabbit antibody was applied at a dilution of 1:200. Sections were incubated with ABC reagent (Vector Laboratories, Inc., Burlingame, CA) for 30 min and then processed for horseradish peroxidase/3,3'-diaminobenzidine tetramethyl chloride (DAB) using the ABC elite system (Vector Laboratories) according to the manufacturer's instructions. Slides were counterstained with hematoxylin followed by ethanol dehydration, and then cleared in xylene and mounted in Permount (Vector Laboratories, Inc.).

Micro-CT and bone histomorphometry

The biomechanical and morphological consequences of osteoblastic and osteocytic AR elevation in AR-transgenic animals were evaluated in 8-wk-old male and female mice (n = 7–14). Right femurs from each genotype were examined for diaphyseal cross-sectional morphology and mineralization using micro-CT. Bones were placed in small polypropylene tubes and immersed in PBS during the scan. Three-dimensional micro-CT images of the diaphyseal region were obtained using an EVS MS-8 micro-CT system (EVS-GE Medical Systems, London, Ontario, Canada). Scans were performed at 6.62-μm voxel size resolution. Before each bone scan, a calibration scan was performed using a three-point calibration phantom corresponding to the density range from air to cortical bone. Bone and non-bone were differentiated using an adaptive thresholding technique (22). Computer-based stereological analyses of the full three-dimensional data set were performed to characterize femoral cross-sectional morphology using methods described previously (23). From the reconstructed and rendered micro-CT images, a 3-mm region of the mid-diaphysis, corresponding to the typical failure region for four-point bending (see below), was examined for cortical area, polar moment of inertia, and mineral content. Morphological traits were quantified for every transverse plane and averaged. The average mineralization of the diaphyseal region for each bone was determined by converting the grayscale value of each voxel to a mineral density value and then averaging mineral density values over all of the voxels for each respective region.

Fluorochrome-based dynamic histomorphometric measurements of bone formation were determined from cross sections at the femoral diaphysis or from frontal sections through the metaphysis. Distal femurs were fixed in ethanol and embedded in methyl methacrylate without prior demineralization. Cross sections (100 μm) through the central portion of the diaphysis were prepared using an SP1600 saw microtome (Leica Microsystems Inc., Bannockburn, IL) and then polished. Frontal sections (5 μm) through the central portion of the distal metaphysis were also prepared using a Poycut sledge microtome (Leica Microsystems Inc.). Dynamic histomorphometric analyses were carried out using a light/epifluorescent microscope with a charge-coupled device camera interfaced to a semiautomatic image analysis system (Bioquant NOVA 4.00.8b, Bioquant, Nashville, TN). Some sections were also stained with Masson's Trichrome (24) for histological evaluation. Static histomorphometric analysis of trabecular bone was performed from the micro-CT image at the metaphysis with computer-aided analysis using the automated trabecular analysis system (25). All measurements were two dimensional. The terminology and units used were those recommended

TABLE 2. TaqMan primers and probes

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Probe (5'-3')
Col1	GGAAGAGCGGAGAGTACTGGATC	CCATGTTGCAGTAGACCTTGATG	ACCCTAACCAAGGCTGCAACCTGGA
Osterix	TGGCTCGTGGTACAAGGCA	GCATGTCCCACCAAGGAGTAG	AGGCATCTCACCAGGTCCAGGCAA
OC	CCTGCTTGTGACGAGCTATCAG	TTTAGGGCAGCACAGGTCCT	TGGCTTGAAGACCGCCTACAAACGC
Cyclin D1	TCTTTCCAGAGTCATCAAGTGTGAC	CGCAGGCTTGACTCCAGAAG	TCCGTGCCTGCCAGGAACAGATTG
TRAP	AATGCCTCGACCTGGGA	CGTAGTCCTCCTTGGCTGCT	CGCACTCAGCTGTCCTGGCTCAAA
OPG	TGAGTGTGAGGAAGGGCGTTA	GGGTTCCAGCTTGCACCA	AGCTGTCCCCCGGGCTCCG
RANKL	CAAGCTCCGAGCTGGTGAAG	CCAAAGTACGTCGCATCTTGATC	AGCATTCAGGTGTCCAACCCTTCCCT
CatK	CCATATGTGGGCCAGGATG	AGGAATCTCTCTGTACCCTCTGCA	TGTATGTATAACGCCACGGCAAAGGCA

Sequence of primers and probes used for real-time RT-PCR analysis of expression of type I α_1 collagen (ColI), osterix, osteocalcin (OC), cyclin D1, TRAP, OPG, RANKL, and cathepsin K (CatK).

by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (26).

Mechanical testing

After micro-CT analysis, the right femurs were subjected to destructive testing to establish whole bone mechanical properties. To test for differences in whole bone mechanical properties among genotypes, femurs were loaded to failure in four-point bending at 0.05 mm/sec using a servohydraulic materials test system (Instron Corp., Canton, MA). Based on methods described previously [as described by Jepsen *et al.* (27)], all whole bone bending tests were conducted by loading the femurs in the posterior to anterior direction, such that the anterior quadrant was subjected to tensile loads. The load-deflection curves were analyzed for stiffness (the slope of the initial portion of the curve), maximum load, post-yield deflection (PYD), and work-to-failure. PYD, a measure of bone brittleness, was defined as the deflection at failure minus the deflection at yield. Yield was defined as a 10% reduction of the secant stiffness (load range normalized for deflection range) relative to the initial (tangent) stiffness. Work-to-failure was defined as the area under the load-deflection curve. Femurs were tested at room temperature and kept moist with PBS during all loading procedures.

Statistical analysis

All data were analyzed using Prism software (GraphPad Software, Inc., San Diego, CA). Significance of difference between wild-type and AR-transgenic mice was assessed by an unpaired two-tailed t test using Welch's correction. One-way ANOVA was carried out to detect overall differences followed by Neumann Keuls multiple-comparison test to calculate intergroup differences. Body lengths and weights were analyzed by repeated measures two-way ANOVA for the effects of gender and genotype. All data are expressed as mean ± SEM.

Results

Generation of transgenic mice

AR-transgenic mice were created with full-length rat AR under the control of the 3.6 kb type I collagen promoter (Fig. 1). The colAR transgene was cloned as described in *Materials and Methods*, using full-length rat AR cDNA and rat type I α_1 promoter sequence from −3518 to +115. To create col3.6 AR-transgenic mice, the linearized colAR transgene was microinjected into pronuclei of fertilized oocytes from B6D2F1 mice, and then transferred into pseudopregnant mice (performed by the OHSU Transgenic Core Facility). Positive founders were identified by genotyping by PCR with primers at locations indicated in Fig. 1A. Founder mice were bred to wild-type B6D2F1 mice; two AR-transgenic lines (lines 104 and 106) derived from independent founders have been retained. Southern analysis confirmed a single insertion site for the AR transgene (Fig. 1B), with five to seven copies of the transgene in each line as determined by real-time PCR quantitation (Fig. 1C). Table 1 lists quantitative real-time RT-PCR analysis of expression of the colAR transgene in various tissues, showing bone targeting with highest levels in calvaria but approximately 100- to 500-fold lower in muscle, skin, heart, intestine, kidney, liver, lung, and spleen. These mice, therefore, display the expected bone-targeted AR expression, consistent with the expression patterns observed by other investigators employing this promoter construct for the generation of transgenic mice (for example, see Refs. 28 and 29).

Phenotype in AR-transgenic mice with bone-targeted AR overexpression

We first determined the effect of bone-targeted AR overexpression on body weight gain and nose-rump length over a 6-month period. At birth, animals were indistinguishable. However, as the mice aged, AR-transgenic males were significantly shorter and weighed less than wild-type littermates (Fig. 2, A and B). In contrast, AR-transgenic females were no different from wild-type controls (Fig. 2, C and D).

We next determined serum estrogen and testosterone levels at 8 wk of age by RIA and enzyme immunoassay, respectively, and as expected, levels were not significantly different between littermate controls and AR-transgenic animals (Fig. 3, A and B). Testosterone levels show some variation because levels rise with puberty in males. There were also no significant differences in serum calcium levels between AR-transgenic and littermate controls for either gender (Fig. 3C), analyzed colorimetrically by the cresolphthalein-binding method. Serum osteocalcin levels were determined by enzyme immunoassay. Interestingly, there was a highly significant, approximately 50% decrease in serum osteocalcin levels in male AR-transgenic animals ($P <$ 0.001, Fig. 3D). A much more modest but also significant decline was observed in female AR-transgenic mice ($P <$ 0.05, Fig. 3D). Serum OPG levels were also analyzed, demonstrating a modest increase in AR-transgenic males, but in contrast a significant decline in AR-transgenic females compared with littermate controls ($P <$ 0.01, Fig. 3E).

Morphological changes in calvaria from AR-transgenic 8-wk-old animals were characterized in fixed, decalcified, and paraffin-embedded calvarial sections from two independent families (104 and 106) after H&E staining (Fig. 4A). It is noteworthy that the calvaria demonstrate thickening that is most dramatic in the male AR-transgenic animals. Most of the new bone formed appears on the periosteal surface of the calvaria in the AR-transgenic mice. Because this phenotype is observed in two independent AR-transgenic lines, it is not likely due to position effects. We next evaluated AR protein expression *in vivo* by immunocytochemical analysis. Nonspecific binding was blocked with normal goat serum, then slides were incubated with AR antibody. Immune complexes were detected after DAB staining, and slides were counterstained with hematoxylin. AR is brown, and the nucleus is bluish purple after counterstaining; immunostaining represents both endogenous AR and the product of the colAR transgene. The majority of osteoblasts and osteocytes demonstrated AR immunoreactivity. Both male and female AR-transgenic animals revealed higher level of AR expression as expected, with no notable difference between the genders (Fig. 4B).

Altered bone morphology with increased trabecular bone volume in AR-transgenic mice

To characterize the quality of bone in the AR-transgenic mice, we examined femoral structure by contact radiography and micro-CT. Overall geometry is shown with faxitron imaging in Fig. 5A. In the AR-transgenic males, the striking bulge near the metaphysis (indicated by the *arrow*), and the changes in cross-sectional morphology, demonstrate that
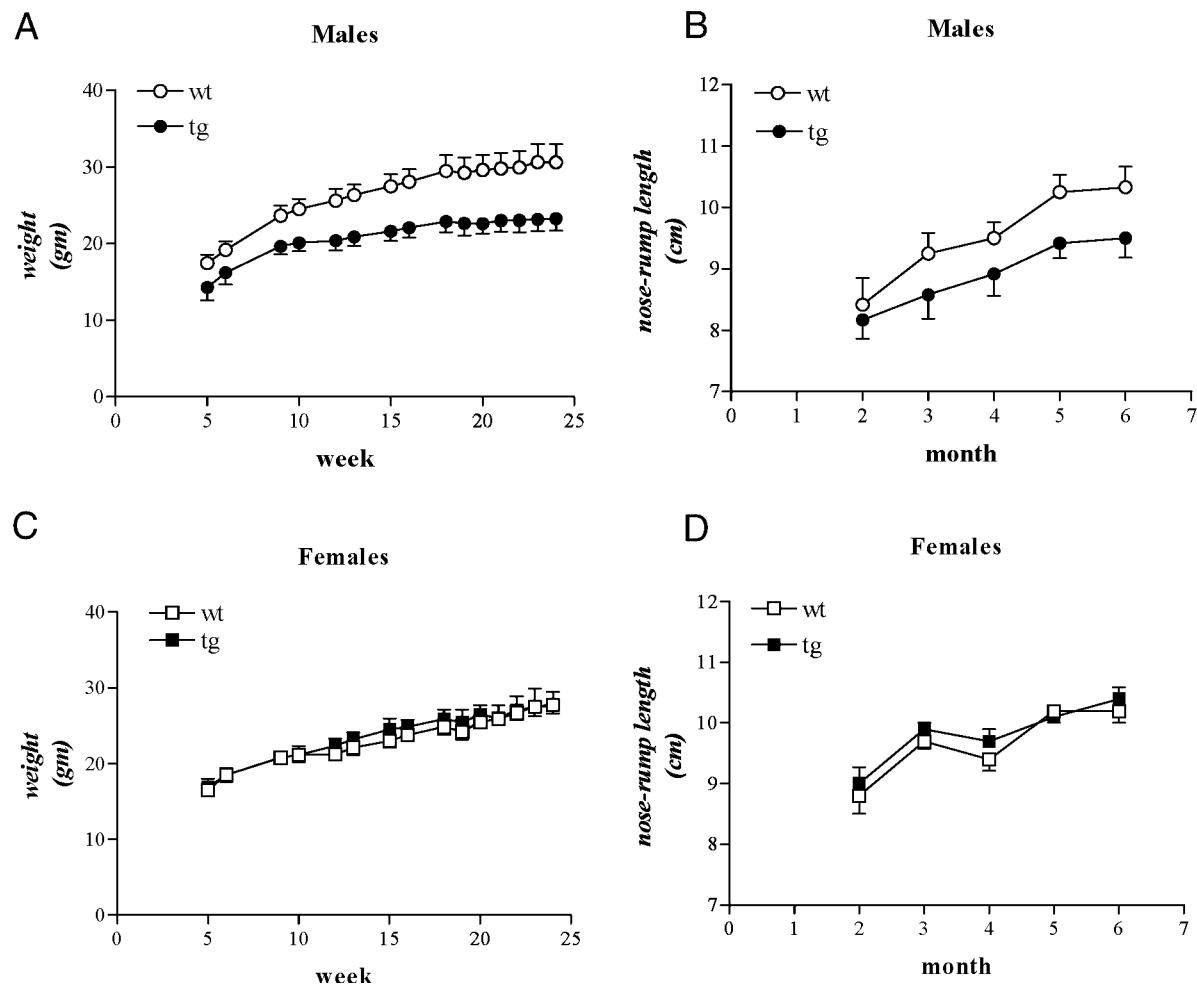
FIG. 2. Age-related changes in body weight and nose-rump length in AR-transgenic mice. Body weight and nose-rump-length determinations were carried out weekly or monthly, respectively, over 6 months in both genders in both wild-type (wt) and col3.6 AR-transgenic (tg) mice (n = 4–5). A, Weight gain in growing male mice. Analysis for the effects of time and genotype by repeated measures two-way ANOVA revealed an extremely significant effect of genotype (F = 54.57; $P < 0.0001$) and time (F = 36.51; $P < 0.0001$) with no interaction. B, Nose-rump length in male mice. Analysis revealed a significant effect of genotype (F = 6.21; $P < 0.05$) and an extremely significant effect of time (F = 15.95; $P < 0.0001$) with no interaction. C, Weight gain in female mice. In contrast to the male mice, analysis revealed no effect of genotype but an extremely significant effect of time (F = 31.32; $P < 0.0001$) with no interaction. D, Nose-rump length in female mice. Again in contrast to the male mice, analysis revealed no effect of genotype but an extremely significant effect of time (F = 25.56; $P < 0.0001$) with no interaction. All data are expressed as mean ± SEM.

normal modeling/remodeling events have been altered. High-resolution micro-CT was also used to evaluate bone morphology in 8-wk-old mice. The reconstructed images (Fig. 5A, *right panels*) also show clear differences in femoral length, cortical thickness at the diaphysis, and bulging at the metaphysis (noted by the *asterisk*) in male AR-transgenic mice. The most dramatic difference in the micro-CT images was noted in trabecular microarchitecture in male AR-transgenic mice, with bulging at the metaphysis (indicated by the *asterisk*), and trabeculae appeared more numerous with the spicules smaller than wild-type controls. The apparent increase in trabecular bone volume in the metaphyseal region of AR-transgenic mice was observed at both the proximal and distal metaphyses. This morphological difference at the metaphysis was not observed in female AR-transgenic mice, consistent with other observations that indicate that the female phenotype is mild at best. These results demonstrate

that AR overexpression has dramatic effects on overall femoral size and shape, and trabecular architecture in male mice. Based on the modest bone phenotype in the female transgenic animals at 8 wk of age, the dramatic changes in male AR-transgenic mice are likely driven by AR transactivation associated with increased testosterone levels that occur as the males fully enter puberty (5–8 wk). Patterns of bone formation were analyzed in more detail by histological methods using femurs of wild-type and AR-transgenic mice. Femurs were embedded in methacrylate without prior demineralization. Frontal sections through the central portion of the distal methaphysis were prepared and stained with Masson's Trichrome (Fig. 5B). Hemotoxylin (*black*) stains nuclei, analine blue (*blue*) stains mineral, Biebrich Scarlet (*red/purple*) stains cytoplasmic elements. Trabecular bone (visualized as *blue stain*) at the metaphysis appears more abundant in the AR-transgenic animals.
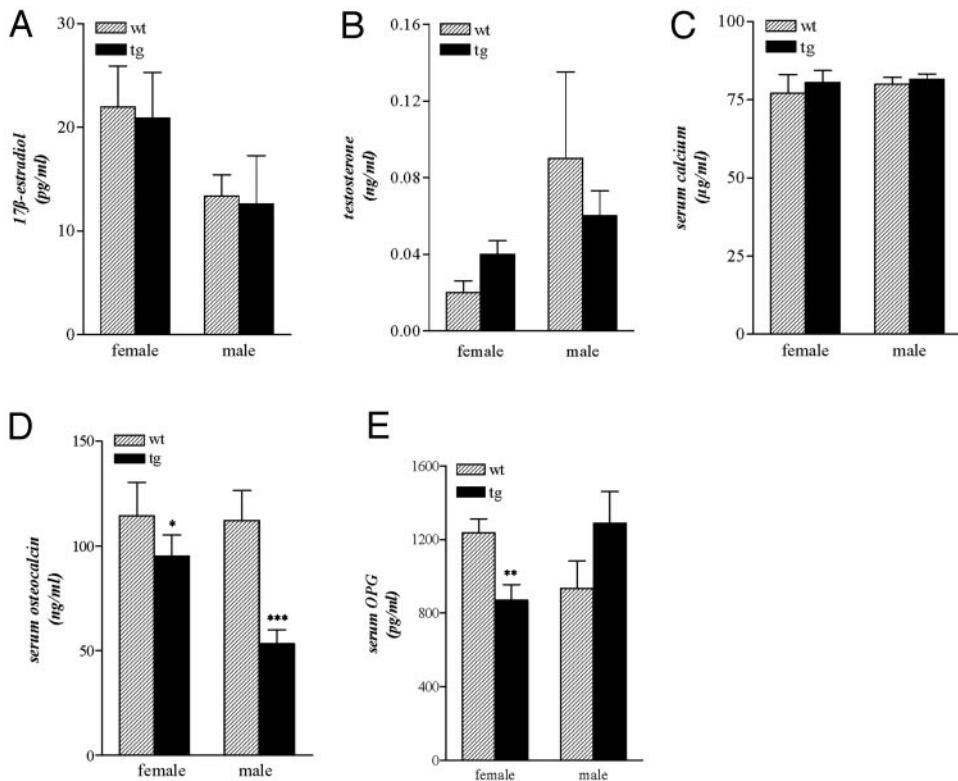
FIG. 3. Biochemical analyses of parameters of calcium metabolism and hormone levels in AR-transgenic animals. Comparisons were performed between littermate control (wt) and AR-transgenic (tg) animals. Serum from 8-wk-old mice (n = 2–7) was analyzed to determine levels of markers of calcium metabolism. Assays were performed in duplicate by RIA for 17β-estradiol, and by EIA for testosterone and intact mouse osteocalcin, and for calcium by the colorimetric cresolphthalein-binding method. A, 17β-estradiol; B, testosterone; C, calcium. There were no statistical differences between 17β-estradiol, testosterone or calcium levels. D, Osteocalcin levels were significantly reduced in both male and female AR-transgenic mice. E, OPG circulating levels were elevated in males but significantly reduced in female AR-transgenic mice. Values are expressed as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (*vs.* gender-appropriate wild-type control).

To examine the mechanism underlying the altered trabecular morphology, the metaphyseal trabecular region in male AR-transgenic mice was analyzed by static (Table 3) and dynamic histomorphometry (Fig. 5C). Static histomorphometric parameters were determined with computer-aided analysis of the micro-CT image to characterize trabecular microanatomy and architecture. Male AR-transgenic mice showed a 73% and 104% increase, respectively, in trabecular bone volume as a percent of tissue volume and bone surface to volume ratio (BV/TV and BS/TV), consistent with the micro-CT and x-ray images (Fig. 5A) and the histological analysis (Fig. 5B). The increase in trabecular bone volume was associated with a 104% increase in trabecular number (Tb.N), a 55% decrease in spacing (Tb.Sp) with a small 16% decrease in trabecular thickness (Tb.Th). There was also a dramatic 306% increase in trabecular junctions (NNd), and a 146% increase in termini (NTm) in the male AR-transgenic mice. Differences in all these measures were statistically significant ($P < 0.05$). Interestingly, several indirect indices of connectivity including the node:terminus ratio (NNd:NTm) and trabecular pattern factor (Tb.PF) were no different between wild-type and AR-transgenic mice.

Bone formation in trabecular bone at the metaphysis was then characterized with dynamic histomorphometric analysis (Fig. 5C). Fluorochromes were administered by double-label injection, with oxytetracycline followed by calcein to label deposition at the mineralizing front. Parameters of bone formation and turnover were reduced in male AR-transgenic mice, including significant reductions in both mineralizing surface as a percent of bone surface (MS/BS; $P < 0.01$) and bone formation rate (BFR; $P < 0.05$). Thus, AR overexpres-

sion in males resulted in increased trabecular volume and a dramatic alteration in trabecular morphology.

Altered cortical bone formation in AR-transgenic mice

Because of the changes in cortical bone observed in the micro-CT images, fluorescent imaging was also carried out at the femoral diaphysis as described for trabecular bone in Fig. 5C. Figure 6A shows patterns of bone formation in images of fluorochrome labeling from femoral cross sections. The AR-transgenic males (*right panel*) demonstrate both a dramatic lack of labeling at the endosteal surface and an increase on the anterior periosteal surface compared with wild-type controls (*left panel*). Consistent with these fluorescent images, dynamic histomorphometric analysis (Fig. 6B), demonstrated divergent responses at the endosteal and periosteal surfaces. MS/BS at the endosteum was dramatically inhibited in AR-transgenic mice ($P < 0.05$), analogous to the reduced bone turnover noted in trabecular bone (Fig. 5C). In contrast to these findings, a nonsignificant increase in formation was noted at the periosteal surface. Similar responses were seen in BFR, with trends for inhibition at the endosteal surface and stimulation at the periosteal surface. Finally, mineral apposition rate (MAR) was dramatically inhibited at the endosteal surface in male AR-transgenic males ($P < 0.05$).

Because of the alterations noted in the fluorochrome images and cortical dynamic histomorphometry (Fig. 6, A and B), and also at the cortical envelope with micro-CT analysis, we also evaluated mineral content, cortical bone volume and polar moment of inertia in the femur mid-diaphysis. As shown in Fig. 6C, both male and female AR-transgenic mice have normal mineral content compared with wild-type lit-
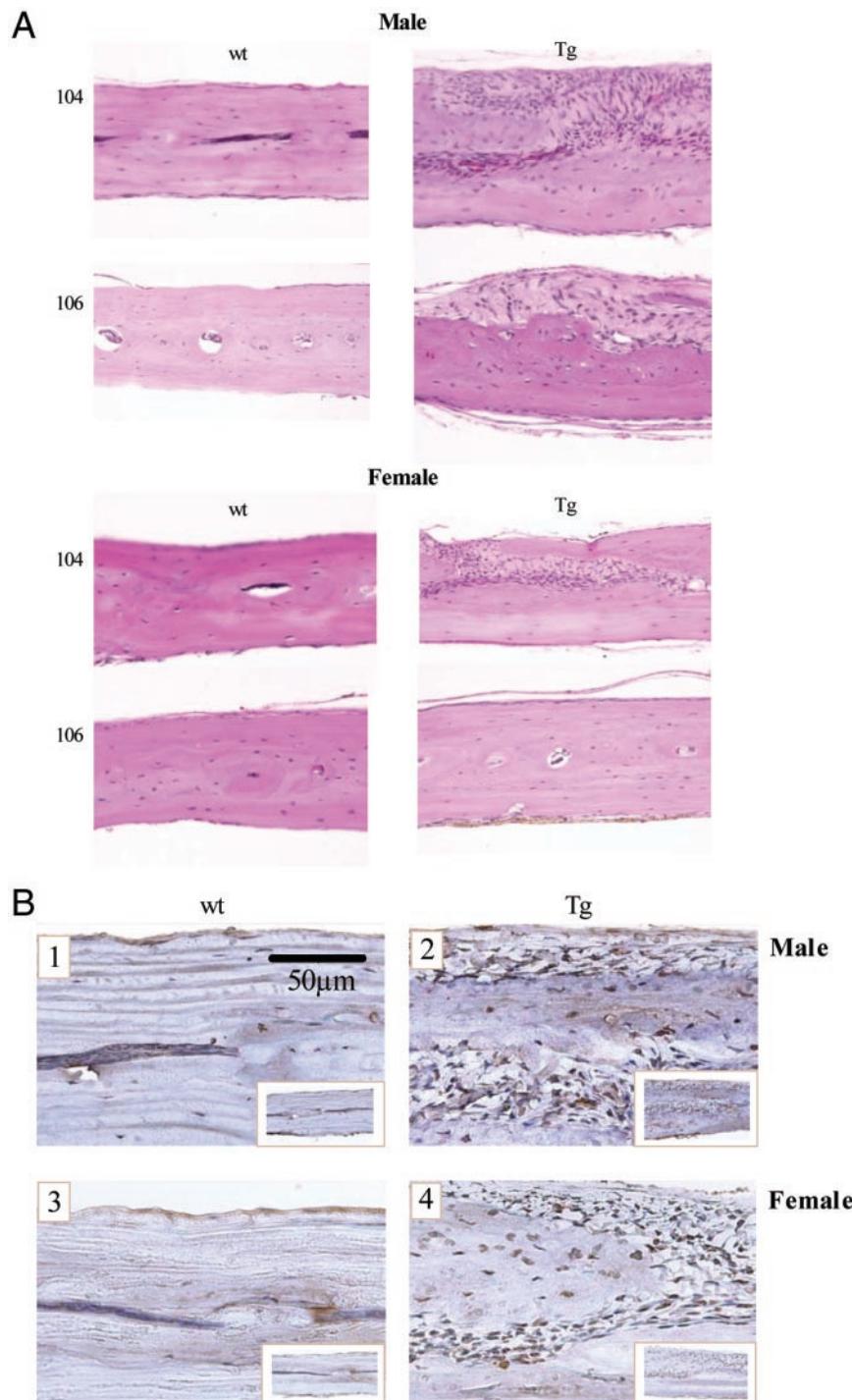
FIG. 4. Histochemical features and immunohistochemical analysis of AR levels in calvaria from AR-transgenic mice. Calvaria were isolated from 8-wk-old male and female mice from both AR-transgenic (AR-tg) lines (104 and 106) and wild-type (wt) littermate controls, and 5-μm sections were subjected to either H&E staining or immunocytochemical analysis after demineralization and paraffin embedding. Representative sections are shown. A, Calvaria from both 104 and 106 families were evaluated. Only male AR-tg animals from both AR-tg families exhibit an increase in calvarial thickness. B, AR was detected with rabbit polyclonal antisera after DAB incubation. AR is brown and the nucleus is *purple* after counterstaining with hematoxylin. The majority of osteoblasts and osteocytes demonstrated AR immunoreactivity ($\times$63). As shown in the *inset* image, there were no observable differences in overall AR expression between male and female animals ($\times$40). *Bar*, 50 μm.

termate controls, but males show a reduction in cortical area ($P < 0.01$) and moment of inertia ($P < 0.05$) consistent with the reduction in labeling at the endosteal surface (Fig. 6, A and B). The female AR-transgenic showed a trend for a slightly smaller cortical area and a reduced moment of inertia ($P < 0.05$).

The dramatic alteration in fluorescent labeling and lack of cortical drift noted in the male AR-transgenic animals could result in changed bone shape. To evaluate bone shape, I_{AP} was divided by I_{ML} [rectangular moments of inertia about the

anterior/posterior (AP) and medial/lateral (ML) axes as gross measures of shape relative to the AP and ML axes]. Each of these terms measures the distribution of bone about these axes; thus, the ratio of these terms provides a measure of shape (the closer the ratio is to 1, the more round the shape, and the further from 1, the more elliptical the structure). Wild-type and AR-transgenic animals were the same for both males and females (data not shown). Thus, the variation in growth patterns may have affected size but not shape during development. The fluorochrome labels are representative of
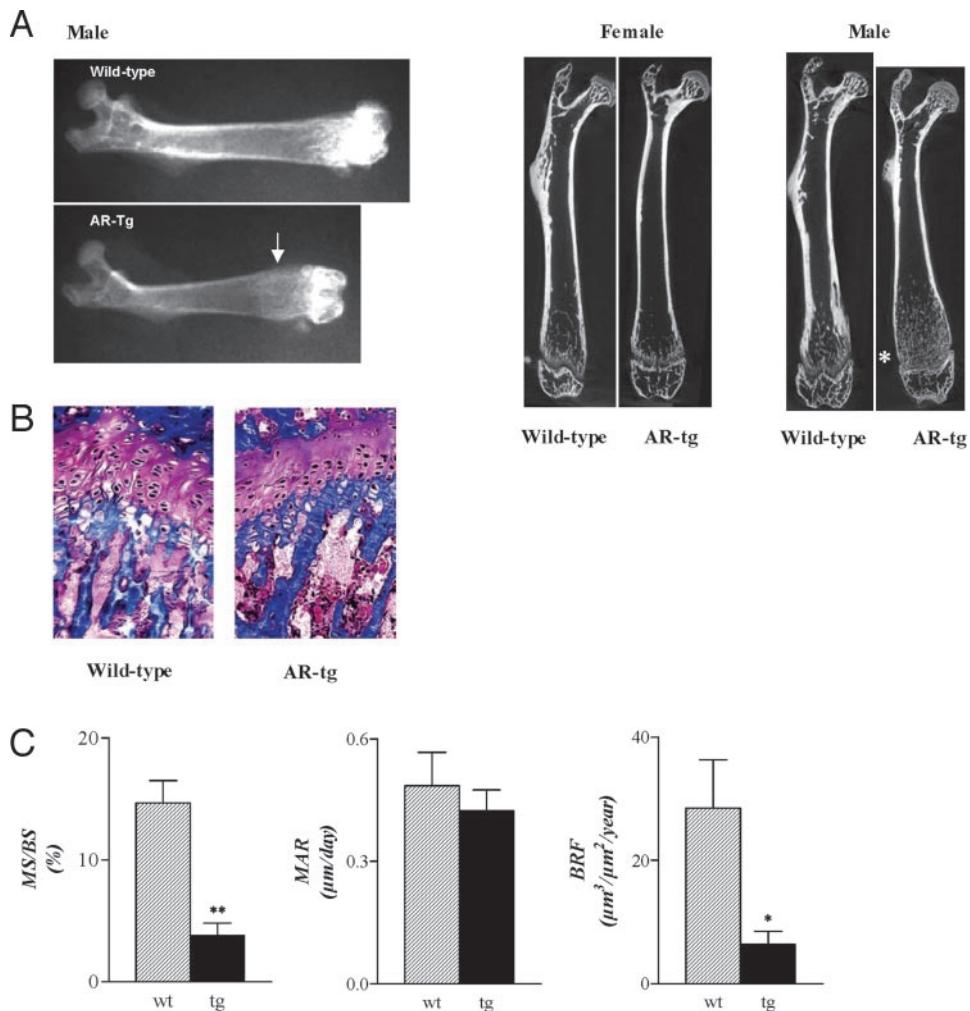
FIG. 5. Radiographic and micro-CT analysis of bone with characterization of trabecular bone formation. A, Femurs were isolated from 8-wk-old male wild-type (wt) or AR-transgenic mice (AR-tg), and subjected to faxitron x-ray imaging. Differences observed in the AR-transgenic male animals (A, *left lower panel*) include bulging at the metaphysis indicated with *white arrow*, and a reduction in femur length compared with the wild-type littermate control (A, *left upper panel*). No difference was apparent in female AR-transgenic or littermate control animals (not shown). *Right panels* show mid-diaphysis of femurs from 8-wk-old male and female AR-transgenic and littermate control mice subjected to high-resolution micro-CT imaging. Mid-saggital section taken from three-dimensional reconstructions of representative mouse femurs from AR-tg and wt littermate controls. Male AR-transgenic mice show altered trabecular bone morphology as indicated by the *asterisk*. B, Histological analysis of metaphyseal bone in AR-tg mice. Femurs were isolated from 8-wk-old male wt or AR-tg, sectioned (5 μm) through the central portion of the distal metaphysis and then stained with Masson's Trichrome. C, Dynamic histomorphometric analysis was performed in trabecular bone after fluorescent imaging microscopy. Surface labeling and mineral apposition were then determined to characterize bone formation (n = 6). Mineralizing surface as a percent of bone surface (MS/BS) and BFR were significantly inhibited in trabecular bone. MAR was not affected in AR-tg males. *, $P < 0.05$; **, $P < 0.01$ (*vs.* wt controls).

mineralization patterns only for the period of time that the labels are present; thus, the patterns we observed likely do not reflect drift/modeling during early development.

Bone strength in AR-transgenic mice is reduced

To analyze bone quality, whole bone failure properties were determined by loading femurs to failure in four-point bending at 0.05 mm/sec. Analysis shown in Fig. 7 revealed significant differences between wild-type and male AR-transgenic animals (family 104) in whole bone biomechanical properties at 8 wk. Male AR-transgenic mice showed significant and relatively large decreases in maximum load ($P < 0.01$, Fig. 7A). A much more modest but also significant decline in female AR-transgenic mice ($P < 0.05$, Fig. 7A) was

also seen. The effect on the female AR-transgenic mice is subtle with only a 13–14% reduction in maximum load when values are body weight corrected. In all other measures, female AR-transgenic mice were not significantly different. In comparison to wild-type and AR-transgenic females, male AR-transgenic mice showed a significant decrease in stiffness ($P < 0.05$, Fig. 7B), PYD ($P < 0.05$, Fig. 7C) and work-to-failure ($P < 0.01$, Fig. 7D). Furthermore, male AR-transgenic mice weighed less and had significantly shorter femur length ($P < 0.001$, Fig. 7, E and F) consistent with the faxitron and micro-CT image in Fig. 6A, indicating that AR action may also have a role in determining closure at the epiphysis or other aspects of longitudinal growth. Similar results were obtained with AR-transgenic family 106.

TABLE 3. Trabecular static histomorphometry analysis

Histomorphometric parameter	wt (n = 3)	AR-tg (n = 4)	*P* value
BV/TV	0.090 ± 0.010	0.156 ± 0.017	0.023 (+73%)
BS/TV (mm/mm^2)	4.713 ± 0.614	9.620 ± 0.884	0.006 (+104%)
BPm (mm)	8.753 ± 1.139	17.865 ± 1.642	0.006 (+104%)
BAr (mm^2)	0.167 ± 0.019	0.290 ± 0.032	0.023 (+74%)
Tb.N (mm^{-1})	2.828 ± 0.368	5.772 ± 0.530	0.006 (+104%)
Tb.Sp (mm)	0.337 ± 0.044	0.153 ± 0.020	0.035 (−55%)
Tb.Th (mm)	0.032 ± 0.001	0.027 ± 0.001	0.026 (−16%)
Tb.Wi (mm)	0.038 ± 0.001	0.032 ± 0.002	0.026 (−16%)
Tb.PF (mm^{-1})	15.961 ± 0.822	17.726 ± 1.163	n.s.
NNd (field^{-1})[a]	3.344 ± 0.839	13.583 ± 3.101	0.041 (+306%)
NTm (field^{-1})	44.789 ± 3.620	110.042 ± 11.569	0.008 (+146%)
NNd:NTm	0.075 ± 0.014	0.122 ± 0.026	n.s.

Micro-CT imaging and computer-aided analysis was used to derive measures of trabecular bone architecture and structure in the femoral metaphysis in male wild-type (wt) littermate control and AR-transgenic (AR-tg) mice. Measurements included trabecular bone volume as a percent of tissue volume and bone surface to tissue volume ratio (BV/TV, BS/TV); bone perimeter and area (BPm, BAr); trabecular number, spacing, thickness, and width (Tb.N, Tb.Sp, Tb.Th, Tb.Wi); and trabecular characteristics including node and terminus number, node:terminus ratio and trabecular pattern factor (NNd, NTm, NNd:NTm, Tb.PF).

[a] Standardized to an arbitrary field size TAr (mm^2), set at 1.857 for all samples. Values are expressed as mean ± SEM. Unpaired, two-tailed *P* values were by Student's *t* test. n.s., Not significant.

Analysis of gene expression in long bone

Finally, we analyzed gene expression in long bone in wild-type and AR-transgenic mice from both genders. Differences in gene expression in RNA isolated from tibial mid-diaphysis were determined by quantitative real-time RT-PCR. Analysis of expression included genes important in both osteoblast and osteoclast activity/development (Fig. 8). Osteoblast genes evaluated were type I collagen, osterix, osteocalcin, and cyclin D1. Osteoclast genes were cathepsin K, OPG, RANKL [receptor activator of nuclear factor-κB (RANK) ligand] and tartrate-resistant acid phosphatase (TRAP). Primer sequences are listed in Table 2. A general inhibition of gene expression in mid-diaphyseal tissue was observed in the osteoblast and osteoclast genes analyzed in both genders of AR-transgenic mice even though the females only have a mild phenotype, consistent with reports that regulation of gene expression by steroid is more sensitive than for other biologic responses (30). Interestingly, male AR-transgenic mice show an increase in OPG levels, whereas females do not, consistent with results from analysis of serum levels (Fig. 3E). The reduction in osteocalcin gene expression was also consistent with changes in serum concentrations (Fig. 3D). In addition, we performed the same analysis with RNA isolated from calvaria and have obtained similar gene expression differences compared with the profiles observed from long bone samples, particularly with respect to the male-specific increase in OPG mRNA levels (data not shown). Thus, there is little difference in the response between the two bone sites as a consequence of AR overexpression. Primers designed to detect both AR and colAR demonstrated an approximately 4-fold elevation in total AR mRNA in AR-transgenic bone in both genders (data not shown), consistent with increased AR protein expression detected with immunocytochemical analysis (Fig. 4B). Finally, we characterized aromatase gene expression in AR-transgenic mice from tibial RNA samples. Although aromatase mRNA expression was readily quantified in positive controls from testis, there were only extremely low levels of aromatase mRNA present in the tibia, and there were no detectable differences in the low levels of aromatase mRNA between male AR-transgenic and wild-type animals (data not shown).

Discussion

AR-transgenic mice were developed with full-length AR under the control of the 3.6 kb type I collagen promoter. Bone-targeted AR overexpression results in a complex phenotype in growing animals. High-resolution micro-CT demonstrated normal mineral content in both male and female AR-transgenic mice, and there are few observable phenotypic differences between the female AR-transgenic mice and littermate controls at 8 wk. Nevertheless, an indication of AR transgene activation may be seen with the modest but statistically significant reductions in serum osteocalcin levels, maximum loading, and polar moment of inertia. In contrast to this mild phenotype in females, male AR-transgenic mice display a complex phenotype with reduced body weight gain and skeletal morphological differences that include widening of the calvaria, altered trabecular architecture with expansion/clubbing at the metaphysis, enhanced periosteal but strongly inhibited bone formation at the endosteal surface, and shorter femoral bones not observed in the AR-transgenic females. In addition, bone quality was impaired in male AR-transgenic mice as demonstrated by a significant inhibition of biomechanical indices including reductions in stiffness, maximum load, PYD, work-to-failure, cortical area, and moment of inertia. AR overexpression during growth thus results in a low-turnover state with increased trabecular bone volume and anabolic stimulation at the periosteum and in calvaria, but reduction of cortical bone due to inhibition at the endosteal surface with reduced turnover in trabecular bone. Collectively, the skeletal phenotype observed in male *vs.* female AR-transgenic mice is likely dependent on higher levels of androgen in males.

The col3.6 promoter was chosen for several reasons: the skeletal expression patterns for this promoter are both well characterized and bone selective (for example, see Refs. 31–33); the col3.6 promoter is active in the periosteum (29), a site of particular interest for androgen responsiveness; and an-
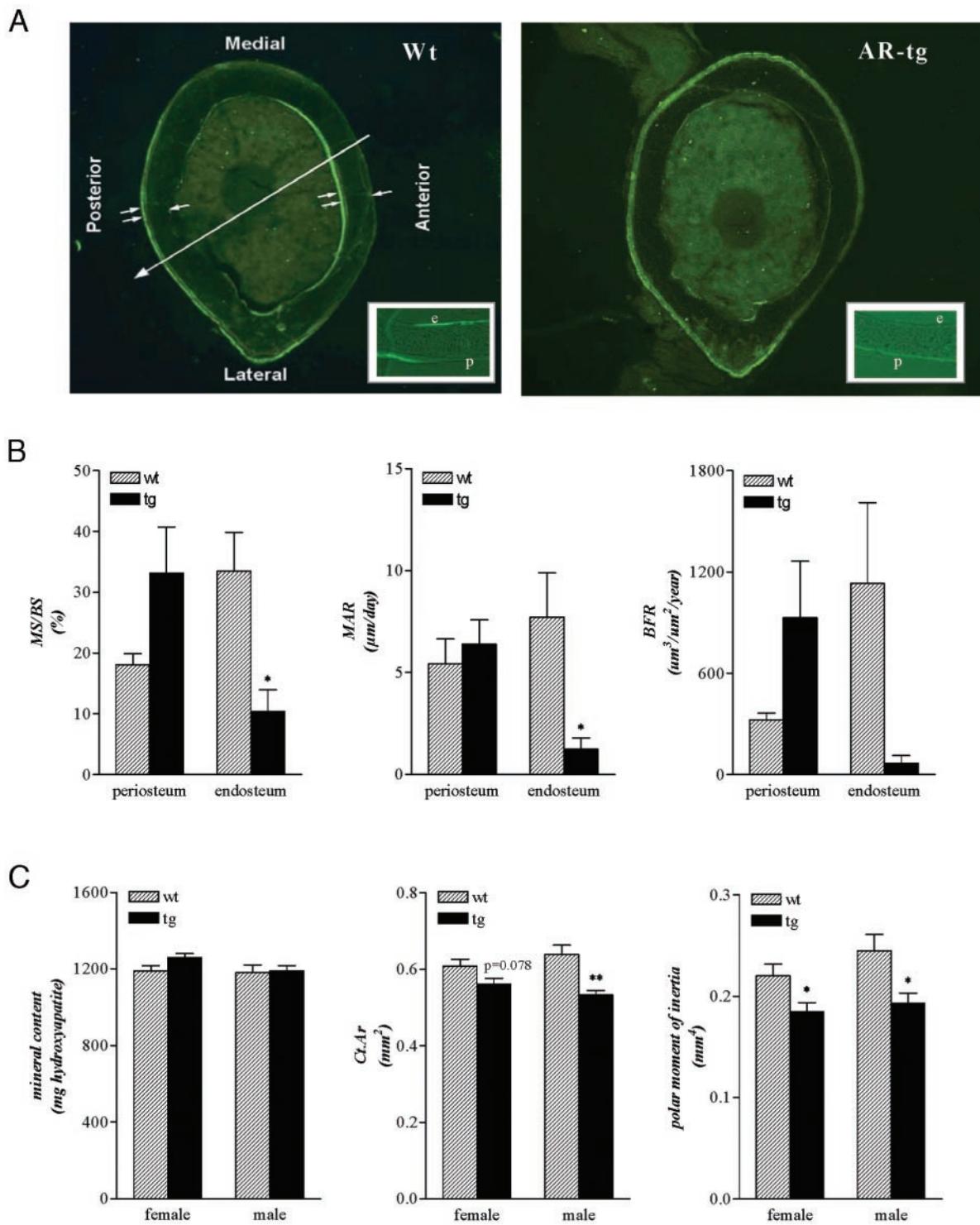
FIG. 6. Characterization of cortical bone formation in AR-transgenic (AR-tg) mice. A, Fluorescent images of femur after double-label administration. Eight-week-old male AR-tg mice were pulsed with oxytetracycline followed 7 d later with calcein to fluorescently label mineralizing surfaces. Femurs were isolated and sectioned (100 μm) at the mid-diaphysis. Sections were subjected to fluorescent imaging microscopy to determine patterns of bone formation. Representative photomicrographs are shown, with sites of formation indicated by *double arrows*. Bands were photographed at comparable anatomic positions for each bone. In contrast to male wild-type (wt) mouse femur, the AR-tg demonstrates a dramatic lack of labeling at the endosteal surface and an increase on the anterior periosteal surface. *Insets* are higher power images demonstrating labeling on the endosteal (e) and periosteal (p) surfaces. B, Dynamic histomorphometric analysis was performed in cortical bone after fluorescent imaging microscopy in AR-tg males (n = 6–8). MS/BS, MAR, and BFR at both the endosteal and periosteal surface were determined in wt and AR-tg mice. C, Mineral content, cortical area (Ct. Ar.) and polar moment of inertia (Jo) (all measured in micro-CT image analysis at the diaphysis) in wt and AR-tg mice. All measures were weight adjusted. Data are mean ± SEM n = 6–9 females, 3–7 males. *, $P <$ 0.05; **, $P < 0.01$ (*vs.* wt controls).
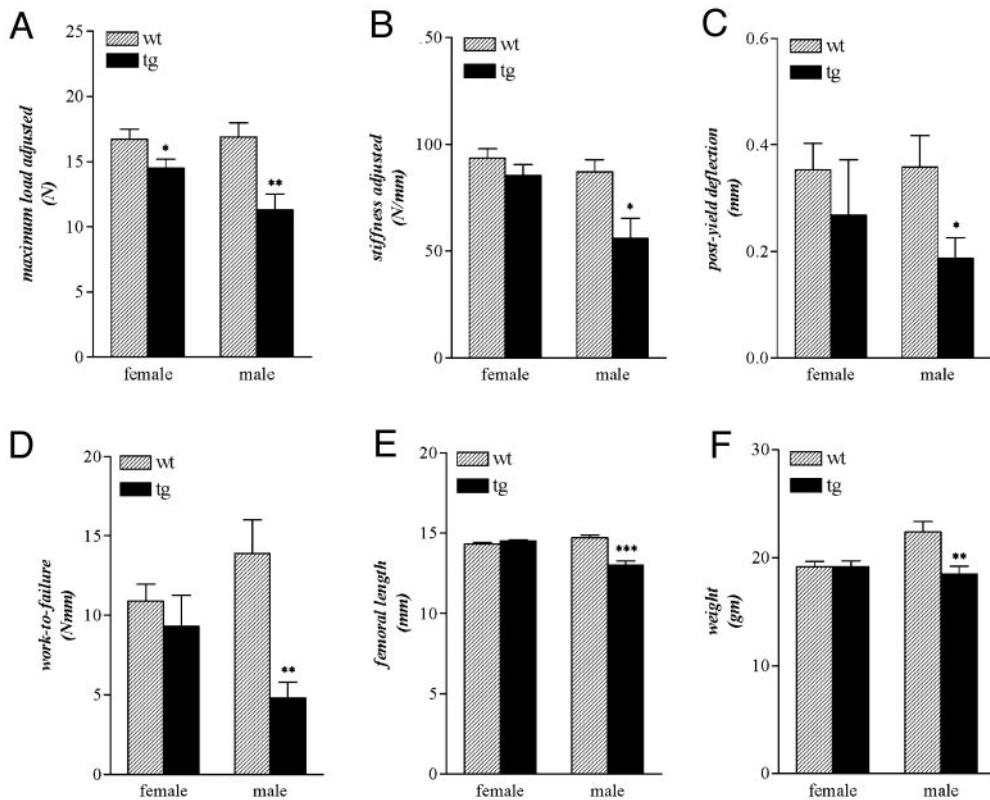
FIG. 7. Biomechanical analyses of bone quality in AR-transgenic mice. Femurs from wild-type (wt) and col3.6 AR-transgenic (tg) mice were isolated from 8-wk-old mice to determine whole bone failure properties. Femurs were loaded to failure in four-point bending and the stiffness, maximum load, and PYD were calculated from the load-deflection curves and adjusted for body weight differences. A, Adjusted maximum load; B, adjusted stiffness; C, PYD; D, work-to-failure; E, femoral length; F, weight. The whole bone biomechanical properties are shown as mean ± SEM, n = 7–14. Differences between genotypes were determined by Student's *t* test. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$ (*vs.* gender-appropriate wt controls).

drogens do not inhibit expression from the 3.6-kb promoter fragment (data not shown). The col3.6 promoter fragment directs expression of the fused transgene very early in periosteal, endosteal, and trabecular surfaces and in cortical bone in the collar region of the growth plate (29). In addition, the col3.6 promoter is more active than the osteocalcin promoter in the control of expression of transgene (32) and is the only known promoter able to drive Cre expression in osteoblasts at a high enough level to induce effective recombination activity (34).

The identification of mechanisms involved in androgen-mediated changes in osteoblast function has important ramifications for both basic and applied knowledge of bone physiology. The discovery of pathways that androgens influence in bone is particularly significant because, as with the recently approved PTH therapy, androgen remains as a promising therapeutic agent with a demonstrated anabolic effect on the skeleton (35). A better understanding of the mechanisms of androgen action in bone will also be relevant for the development of a class of drugs termed selective AR modulators, for the treatment of osteoporosis and other disorders (36). These drugs, analogous to the selective ER modulators (37), are being developed to stimulate anabolic actions in bone, but to not display the detrimental changes in lipid profiles, increases in prostate growth and facial hair growth in women that have been associated negatively with

androgen therapy. Thus, the elucidation of the mechanism(s) mediating the effect of androgens on osteoblasts will provide for a better understanding of the cascade of molecular events associated with androgen exposure in bone, may provide a framework for improvements in the diagnosis and treatment of metabolic bone diseases, and could help to identify approaches to overcome the deleterious consequences of hypogonadism on bone mass. Because of an enhanced responsiveness in the AR-transgenic animals, the AR-transgenic mice represent a useful model to identify important actions of androgen that influence skeletal modeling and remodeling.

Analysis by micro-CT and faxitron imaging demonstrated altered trabecular bone morphology only in the male AR-transgenic mice. A particularly striking "bulge" at the metaphysis is observed, and femur length is reduced. The trabecular tissue phenotype is dramatic and is seen in the metaphyses at both ends of the femur. In the male AR-transgenic animals, static histomorphometric analysis in the metaphysis confirmed the increased trabecular bone volume, comprised of more trabeculae that are smaller in size and closer together. Because bone volume fraction was increased, AR overexpression appears to be beneficial, but further biomechanical analysis will be necessary to confirm that these morphological changes result in superior mechanical properties. These results are consistent with the histological,
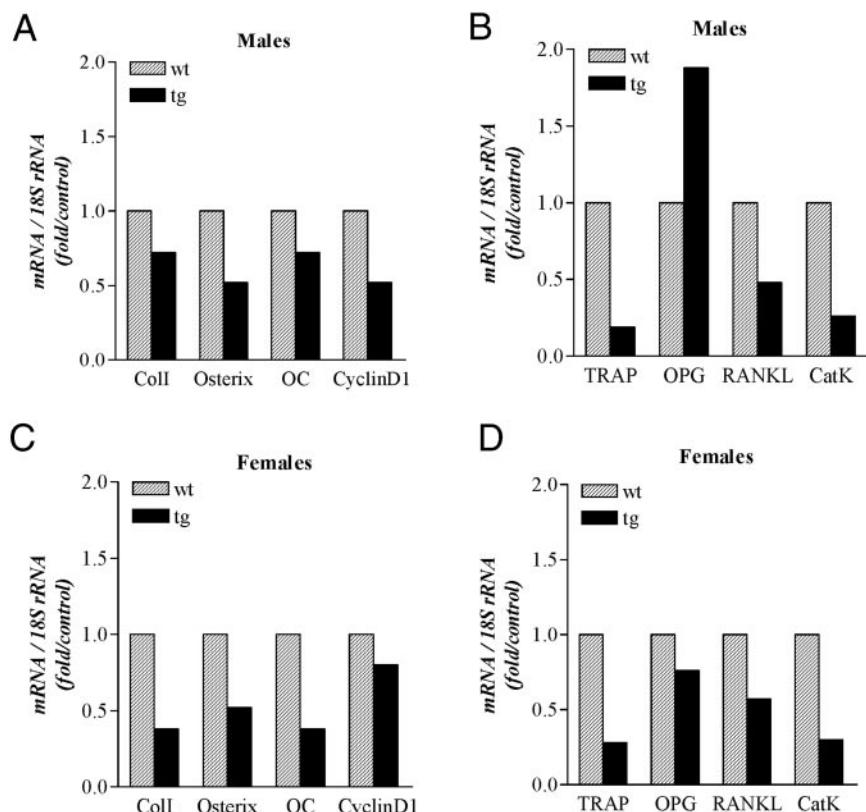
FIG. 8. Gene expression in AR-transgenic mice. Analysis of steady-state mRNA expression of osteoblast or osteoclast genes was determined by real-time RT-PCR analysis using tibial RNA isolated from wild-type (wt) or AR-transgenic mice (tg), n = 5. Osteoblast genes involved in bone formation and matrix production examined included ColI, osterix, osteocalcin (OC), cyclin D1. Osteoclast genes involved in bone resorption were TRAP, OPG, RANKL, and cathepsin K (CatK). A and B, Analysis of expression of osteoblastic (A) and osteoclastic (B) genes in males. C and D, Analysis of expression of osteoblastic (C) and osteoclastic (D) genes in females.

micro-CT, and faxitron images and are analogous to the phenotype observed in growing animals treated with antiresorptive drugs because a similar morphology is observed in both human and rodent growing skeleton after administration of bisphosphonates. These effects of treatment noted in these studies include increased metaphyseal bone probably due to higher trabecular number not thickness, a "club-shaped" metaphysis, reduced surface-based indicators of trabecular bone modeling or remodeling, and decreased femur lengths in some studies (38–40). In the adult skeleton during remodeling, bone resorption and formation are physically closely coupled, and as a consequence both processes would be inhibited with antiresorptive drug treatment. However during modeling in the growing animal, osteoclasts and osteoblasts are active on different and distinct surfaces and are thus uncoupled. It is thus interesting to speculate that AR overexpression in the osteoblast lineage can alter the way trabecular bone is remodeled or constructed in the metaphyseal region during growth.

A possible reduced turnover phenotype in AR-transgenic mice is also suggested from the decline in serum osteocalcin levels, analogous to the observation that DHT treatment reduces osteocalcin levels in gonadectomized rat models (41, 42). In addition, reduced serum osteocalcin levels have been observed with antiresorptive therapy (43). Serum osteocalcin is a complex measure reflecting bone turnover and could represent either decreased formation or decreased resorption or both (44). Because of the resemblance of the phenotype to that seen after antiresorptive drug administration in growing animals described above and because of the increased formation noted in the calvaria and periosteal surface where

there are few osteoclasts, these findings are consistent with an anabolic and/or antiresorptive response in AR-transgenic mice depending on the site. Changes in osteoclastic gene expression with increased OPG and decreased cathepsin K, TRAP, and RANKL levels in AR-transgenic bones are also consistent with suppression of bone resorption in these animals, likely mediated through communication with AR-targeted osteoblastic cells. Evaluation of serum levels of OPG also revealed an increase in males but decrease in female AR-transgenic mice, consistent with the changes observed in gene expression. The effects of androgens on OPG levels are controversial because they have been associated both positively (45) and negatively with androgen levels (46, 47). Furthermore, these results suggest a possible role for the RANKL/RANK/OPG signaling pathway in the AR-transgenic mice and are consistent with reports that androgen inhibits osteoclast activity (48) and osteoclastogeneisis (16, 49, 50). The effect of androgens on osteoclast survival is also controversial, with either enhancement (13) or no effect (16) reported. Ongoing histomorphometric and *in vitro* analyses, designed to characterize osteoblast and osteoclast number and activity, will be helpful in addressing these issues.

We have obtained no evidence that estrogen action plays a role in the phenotype that is observed in the AR-transgenic animals. Because the bone phenotype (size, cortical thickness *etc.*) of the transgenic male is smaller than the wild-type female, the male phenotype is not readily explained by increased transactivation of ER (because activation of ER in the male would not make the male skeleton smaller than the female). There is no evidence that circulating estrogen levels are affected in these animals because the serum estrogen

concentrations are not different between wild-type and AR-transgenic males. It is possible that local aromatization may increase estrogen concentrations in bone without influencing circulating levels. Although both aromatase mRNA and activity have been detected in cultured osteoblasts, treatment with androgen (*i.e.* R1881 or DHEA) had no effect on osteoblast aromatase activity (51). Consistent with this result, we observed no detectable differences in the very low levels of aromatase mRNA present in tibial samples between male AR-transgenic and wild-type animals.

The bone phenotype observed in AR-transgenic mice is also consistent with many of the known effects of androgen treatment on the skeleton. The skeletal response to androgen treatment has been characterized as increased cortical and trabecular bone mass (with increased trabecular number but not thickness), increased bone width with surface periosteal expansion and a lack of inner endosteal deposition, in the setting of inhibition of resorption due to reduced osteoclast activity (9, 20, 52–54). Although AR-transgenic males demonstrate an altered trabecular morphology with increased trabecular bone volume and increased periosteal apposition, they also exhibit dramatic inhibition at the endosteal envelope that may be responsible for the decreased cortical bone area and changes in biomechanical properties we have observed. Not surprisingly, DHT replacement does not preserve mechanical strength in orchidectomized mice (54). Inhibition of osteoclastic resorption may be responsible for bulging at the metaphysis and altered trabecular morphology in AR-transgenic males and would be consistent with reduced osteoclast activity and increased trabecular bone observed with androgen therapy.

Some characteristics of the phenotype observed in 8-wk-old male AR-transgenic mice cannot be explained by the known physiological actions of androgens in adults. For example, biomechanical analysis of the skeletal phenotype demonstrated significantly reduced stiffness, maximum load, PYD, and work-to-failure in male AR-transgenic mice. The reduced stiffness and maximum load are consistent with a reduction in cross-sectional area (27, 55). Significant reductions in PYD and work are indications that bone quality and/or matrix properties may be altered in these mice. The reduced PYD observed in the male AR-transgenic mice cannot be explained by an increase in mineral content because the male AR-transgenic show a similar mineral content value compared with male wild-type by high-resolution micro-CT analysis. Therefore, other changes in bone matrix quality are likely responsible (*e.g.* analysis of MOV13 mouse, see Ref. 56). A potential change in bone matrix quality in AR-transgenic mice is also consistent with the reduced osteoblastic gene expression noted in the tibial RNA samples. Significantly shorter femurs observed in male AR-transgenic mice suggest premature closure at the epiphysis and could represent direct effects in the cartilage because it has been reported that there is a low-level col3.6 transgene activation in hypertrophic cartilage (29). However, this phenotype is unlikely to be a result of direct effects in cartilage because testosterone injections result in stimulatory effects on epiphyseal growth in castrated rats (57). It is also possible that the effects of androgens on epiphyseal closure are due to accelerated differentiation of osteoblasts toward osteocytes, leading to early

ossification and mineralization in the secondary spongiosa. We have previously demonstrated increased AR expression as osteoblasts differentiate into osteocytes, suggesting a role for androgen in osteoblast differentiation (58). Androgen treatment has also been shown to enhance mineralization (59).

Not surprisingly, the overall phenotype observed in the male AR-transgenic mice represents in some ways the converse of that characterized in null AR mice with global elimination of functional AR. With the null AR mutation, mice develop a high turnover osteopenia, with increased formation in trabecular bone and at the endosteal surface, but even higher bone resorption resulting in reduced trabecular volume (16, 17). Conversely, we have demonstrated that with bone-targeted AR overexpression, males develop a phenotype consistent with reduced turnover and inhibition of osteoclast activity, suggested by the changes in osteoblastic and osteoclastic gene expression, and reduced serum osteocalcin levels, elevated OPG levels, and the altered morphology noted at the metaphysis with increased trabecular bone volume but a reduction in MAR. Loss of AR function in the null AR model also increased RANKL expression (16), opposite to the inhibition in RANKL expression we observed in males with AR overexpression. Thus, AR-transgenic mice and AR null mice both have altered bone quality, but likely through different cellular mechanisms.

Evidence of the anabolic actions of androgens can be observed at the calvaria and periosteum. Male AR-transgenic mice develop widening of the calvaria that could reflect periosteal expansion. In addition, enhanced periosteal apposition after fluorochrome labeling in the femur was noted, but with a dramatic reduction in endosteal labeling characterized by decreased MAR and BFR and thus a change in the normal cortical drift pattern at the time of labeling. These data, particularly in cortical bone and at the calvaria, are consistent with the documented anabolic effects of androgens on periosteum. Although the reduction of endosteal apposition could reflect an effect on bone resorption because the marrow space undergoes net bone resorption as the bone is growing, it is also conceivable that transgenic expression of AR suppresses endosteal bone formation. AR transactivation directly in osteoblastic cells may thus play a primary role in determining sexual dimorphism in the skeleton, *i.e.* that male bones tend to be wider but not thicker (20).

In summary, skeletally targeted AR overexpression results in a complex phenotype characterized by increased periosteal bone formation and calvarial thickening, and decreased bone turnover/bone resorption on trabecular and endosteal surfaces, leading to increased trabecular bone volume with increased trabecular number and reduced trabecular separation. In addition, despite the increased periosteal growth, the size and length of long bone, cortical area and mechanical properties are all reduced in male AR-transgenic mice, which may result from inhibition of bone turnover during growth as is observed with antiresorptive therapies. Thus, whereas aromatization of androgen to estrogen does contribute to anabolic responses seen with testosterone (Ref. 41, but also see Refs. 54 and 60), we postulate that the direct effects of androgen on osteoblasts through AR transactivation are nevertheless important. The skeletal phenotype is seen predom-

inantly in male AR-transgenic mice with elevated circulating androgen levels relative to the females, and in many ways resembles that observed with androgen treatment. Therefore, the differences observed between littermate controls and the bone-targeted AR-transgenic lines suggest that many of the effects of androgen therapy are the consequence of direct androgen transactivation of the AR in bone. Analysis of the AR-transgenic mice may thus provide a proof-of-principle that AR transactivation directly in the osteoblastic lineage mediates at least some effects of androgens on skeletal homeostasis.

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Androgens and bone growth: it's location, location, location

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Androgens increase bone mass in specific skeletal compartments through effects on bone cells, enhancing osteoblast activity but inhibiting that of osteoclasts. The mechanism of action of androgens might involve both classic androgen receptor transcriptional activation and rapid non-genomic effects, and could also be dependent upon low levels of estrogen.

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Introduction

Although it is well-established that androgens play a key role in skeletal growth and turnover, the mechanisms remain controversial given that the major androgen metabolite, testosterone, is also a substrate for the production of estradiol through aromatase activity. Additional complications arise with the complex nature of bone itself. There is, however, little controversy regarding anabolic effects of androgens on one skeletal compartment: the periosteal or outside surface of bone. Androgen action at the onset of puberty is responsible for the development of a sexually dimorphic skeleton (i.e. male bones are wider than female) and is a significant determinant of bone strength. Androgens are also important for the production of peak bone mass in males. Cell types known to express androgen receptors (ARs) include osteoblasts, osteoclasts and mesenchymal stromal cells that differentiate toward the osteoblast lineage. Proof-of-concept for AR action is demonstrated in androgen-insensitivity models, with production of a nonfunctional AR associated with a dramatic reduction in total bone mass in humans even with excellent estrogen compliance. AR null mice have high turnover osteopenia with increased formation but even stronger enhancement of resorption. Targeted AR over-expression in the skeleton further supports these findings, as it results in enhanced periosteal formation, reduced endosteal formation, increased trabecular bone formation and a reduction of osteoclast activity, all without changes in circulating steroid levels.

Although both estrogen and androgen circulate in men and women, the influence of each on the remodeling skeleton is distinct as shown by divergent responses to gonadectomy in either gender and during modeling, particularly with respect to bone size (periosteal apposition). Non-parallel pathways of action are also indicated with the observation that combination therapy combining estrogen and androgen is more beneficial than either steroid alone in post-menopausal women [1–3]; this has been confirmed in animal models [4•,5]. Estrogens are thought to maintain adult bone mass predominantly through inhibition of bone resorption by osteoclasts (i.e. they act as anti-resorptive agents), which protect the skeleton from further loss of bone. Non-aromatizable androgens such as 5α-dihydrotestosterone (DHT), conversely, are anabolic agents that increase bone mass by stimulation of bone formation, and thus represent an important therapeutic class that has the potential to re-build lost bone.

The purpose of this review is to discuss recently published data regarding the anabolic effects of androgen on bone growth, with a focus on the mechanisms by which androgen regulates skeletal modeling and remodeling through transactivation of the AR.

The complexity of bone: distinct compartments and cell types

The cell biology that underlies bone development is complex. Two processes are evident in the development of bone: endochondral formation from a cartilage anlage as seen in long bones, and intramembraneous formation seen in calvaria (skull cap). Long bones are organized in compartments of cortical lamellar bone and trabecular spiny or cancellous bone. Cortical bone is formed at midshaft (diaphysis/cortical), whereas trabecular bone is formed at the growing end (metaphysis/trabecular). The cortical bone outer surface is the periosteal compartment, and the inside surface is the endosteum. Periosteal bone formation defines the cross-sectional area of bone, whereas endosteal formation or resorption determines cortical thickness. As described below and summarized in Table 1, the effects of androgen on bone formation are complex, and vary according to the specific skeletal site.

Androgen, the AR and bone cells

Ultimately, bone mass is determined by two processes: formation and resorption. Distinct cell types mediate

Table 1

Androgen effects on bone formation.

Skeletal site	Response
Cortical bone	
Periosteal surface	Increase
Endosteal surface	Decrease
Trabecular bone	
Volume	Increase
Perimeter	Increase
Trabecular number	Increase
Trabecular spacing	Decrease
Trabecular thickness	Decrease/no effect
Intramembraneous bone	
Calvarial thickness	Increase

these processes. The bone-forming cell, the osteoblast, synthesizes bone matrix and regulates mineralization, and is responsive to most calciotropic hormones. The osteoclast is responsible for bone resorption. Although bone is clearly a target tissue with respect to androgen action, the mechanisms of action and cell types by which androgens exert their effects on bone biology are incompletely characterized. An additional complexity in terms of mechanism of action is that androgens can influence bone directly by activation of the AR, or indirectly after aromatization of androgens into estrogens with subsequent activation of estrogen receptors (ERs).

In vivo analysis has demonstrated significant expression of AR in osteoclasts and in all cells of the osteoblast lineage, including osteoblasts and osteocytes [6]. Data show preferential nuclear staining of ARs in males at sexual maturity, suggesting activation and translocation of the receptor in bone when androgenic steroid levels are elevated. Interestingly, ARs are also expressed in mesenchymal precursor cells [7•] — pluripotent cells that can differentiate into muscle, bone and fat. Androgen action can modulate precursor differentiation toward the osteoblast and myoblast lineage, while inhibiting differentiation toward the adipocyte lineage [8]. These effects on stromal differentiation might underlie some of the well-described consequences of androgen administration on body composition [9].

Evidence does suggest that androgens can act directly on the osteoblast. There are reports, some in clonal osteoblastic cell lines, of positive effects of gonadal androgen treatment on differentiation, matrix production and mineral accumulation [3]. Interestingly, the effect of non-aromatizable androgens on osteoblast proliferation is biphasic, with stimulation occurring initially but inhibition being seen with longer treatment times [10••]. This reduction in osteoblast viability, which can be inhibited with AR antagonists, might be associated with enhanced apoptosis, even in settings of enhanced bone formation (K Wiren, unpublished). Androgens may also augment the

osteoanabolic effects of mechanical strain in osteoblasts (see also Update) [11].

Analysis of AR, ERα and ERβ mRNA and protein expression during osteoblast differentiation *in vitro* show that all receptors display expression patterns dependent upon the stage of differentiation [12]. The levels of AR expression increase throughout osteoblast differentiation, with the highest AR levels seen in mature osteoblast/osteocytic cultures, suggesting that androgen may predominantly affect mature osteoblasts. Thus osteoblast differentiation and steroid receptor regulation are intimately associated. Androgen exposure has frequently been shown to upregulate AR expression in osteoblasts [13], suggesting increased AR levels and responsiveness as androgen concentrations increase.

Potential modulation of osteoclast action by androgens is suggested by reports of AR expression in the osteoclast [6]. In addition, androgen treatment reduces bone resorption of isolated osteoclasts, inhibits osteoclast formation [14], including that stimulated by parathyroid hormone [15], and might play a direct role in regulating aspects of osteoclast activity in both AR null mice [16] and AR transgenic mice [17••]. Indirect effects of androgen to modulate osteoclasts are indicated by the increase in osteoprotegerin by testosterone treatment in osteoblasts (see also Update) [18]. Androgen levels are likely to be a less significant determinant of bone resorption *in vivo* than those of estrogen [19,20], although this remains controversial [21].

There has been speculation that the positive effects of androgens on the skeleton may be mediated through non-specific, non-genomic actions [22], including non-genomic AR signaling in osteoblasts [23]. Recent data, however, suggest that genomic signaling may be the more significant regulator in bone [24–26].

The effects of sex steroids in the development of a sexually dimorphic skeleton

During childhood and adolescence, skeletal development is characterized by marked expansion of cortical proportions and increasing trabecular density. During this process, the skeleton develops distinctly in males and females, particularly at the periosteal surface. Sex differences in skeletal morphology and physiology occur at or around puberty, with little effect of gonadal steroids prior to puberty [27]. For that reason, it is hypothesized that gender differences, particularly with respect to 'bone quality' and architecture (i.e. predominantly bone width) are modulated by the sex steroids estrogen and androgen. Thus, a distinct response to estrogen and androgen has been described *in vivo* especially in cortical bone. At the periosteum, estrogen suppresses, whereas androgen stimulates, new bone formation yet, conversely, at the endosteum estrogen stimulates, but androgen strongly

suppresses, formation [17••]. Interestingly, low levels of estrogen (in the obligate presence of androgen) might also be important for stimulation of periosteal bone formation during development [28•]. Young men do have larger bone area than women, particularly at peripheral sites [29]. Thus, estrogen decreases, but androgen increases, radial growth in cortical bone (see also Update). These distinct responses to estrogen and androgen probably play an important role in determining sexual dimorphism of the skeleton (i.e. male bones being wider but not thicker than those of females) [30]. Androgens are also essential for the production of peak total-body bone mass in males [31•].

The importance of androgens in bone formation: human studies

It is also clear that androgens play an important role in maintaining the adult skeleton. Hypogonadism in both sexes is associated with bone loss. Replacement therapy with estrogen, aromatizable testosterone or non-aromatizable androgen (e.g. DHT) are all generally effective at ameliorating this loss. Clinical data demonstrate that estrogen replacement therapy tends to suppress bone resorption with only modest effects on bone formation. By contrast, androgen replacement data have demonstrated clear effects on bone formation. The skeletal response to androgen administration has been characterized as increased cortical and trabecular bone mass, together with increased bone width with surface periosteal expansion but a lack of inner endosteal deposition, in the setting of inhibition of resorption owing to reduced osteoclast activity [19,21,32,33••]. Thus, androgens act to maintain trabecular bone (see also Update) and expand cortical bone. In female-to-male (genetic female) transsexuals, high-dose testosterone therapy resulted in increased bone mineral density (BMD) at the femoral neck, with estradiol declining to post-menopausal levels [34]. In a second study, the BMD of female-to-male transsexuals treated with 'male' levels of testosterone increased to normal male levels at cortical sites [35]. Finally, men with constitutional delay of puberty have impaired periosteal expansion [36•].

The global contribution of ARs to bone quality in humans has been assessed in women with androgen insensitivity syndrome (AIS) with 46,XY genotypes. In such patients, AR abnormalities produce an impaired (to complete) lack of response to endogenous and exogenous androgens. Women with AIS who were rated to have been poorly compliant with estrogen therapy showed significantly lower bone mass (indicated by BMD) than those with good or excellent compliance ratings [37]. However, even those women who 'promptly started and fastidiously maintained estrogen replacement' showed a significant deficit both in lumbar spine BMD and in lumbar and femoral bone mineral apparent density, which largely corrects for the confounding effects of bone size. Thus,

the lack of direct skeletal actions by androgen underlies at least part of the bone mass deficits observed in women with complete AIS. Although aromatization of testosterone to estrogen metabolites can play an important role in mediating the effects of androgens in bone, the significance of AR action independent of ER in mediating androgen effects is clear [38].

Animal studies: androgen administration and AR null and transgenic mice

Results from animal studies also support an effect of androgen on bone formation. Experimental strategies, such as surgical or pharmacological intervention, and examination of genetic models have all been employed to characterize androgen signaling. Distinct effects of androgen are seen with gonadectomy when comparing the effects of orchidectomy (ORX) in male versus ovariectomy (OVX) in female rats. OVX and the associated loss of sex steroids in the female generally results in decreased trabecular area with increased osteoclast number. In cortical bone, an increase in bone formation at the periosteal surface is seen with circumferential enlargement, but a decrease is seen in endosteal bone formation. These results demonstrate that estrogen protects trabecular bone predominantly through inhibition of osteoclast activity/recruitment, but has an inhibitory action at the periosteal surface (for example, see [39••]). In male rats, ORX with the attendant loss of sex steroids also results in decreased trabecular area with increased osteoclast number. However, in contrast with females, periosteal formation in cortical bone is reduced with the loss of androgen. Androgen treatment is effective in suppressing the acceleration of bone remodeling normally seen after ORX [40]. Histomorphometric analysis of androgen action in ORX male mice has shown that the bone-sparing effect of AR activation in trabecular bone is distinct from that of ERα at the same site. Thus, AR activation preserves the number of trabeculae but does not preserve thickness or volumetric density, or mechanical strength in cortical bone [41]. In the intact animal, stimulation of endosteal formation by estrogen compensates for the lack of periosteal formation, resulting in no difference in biomechanical strength between the sexes. Nevertheless, factors that influence periosteal apposition might constitute an important therapeutic class, as periosteal bone formation is often a neglected determinant of bone strength [42]. In addition, it appears that ORX affects cranial development more than OVX [43], suggesting that androgen action is particularly important in intramembranous bone.

The specific contribution of AR signaling *in vivo* has been approached in genetic animal models with global AR modulation, including the testicular feminization (Tfm) model of AIS [39,44] and with non-targeted AR knockout mice [16,45]. In the Tfm model, ORX demonstrates the importance of AR in mediating the positive effects of androgen in maintenance of trabecular bone, and of

cortical bone particularly at the periosteal surface [39,44]. The bone phenotype that develops in a global AR null male mouse model is high-turnover osteopenia, with reduced trabecular bone volume and a significant stimulatory effect on osteoclast function [16,45,46]. As expected, bone loss with ORX in male AR null mice was only partially prevented by treatment with aromatizable testosterone owing to the lack of AR.

A final model for AR modulation is represented by modest overexpression of AR in AR transgenic mice, constructed with full-length AR under the control of the 3.6 Kb type I collagen promoter [17**]. AR transgenic mice are the only model with skeletally targeted manipulation of AR expression, and demonstrate enhanced sensitivity to androgen without changes in circulating steroids or androgen administration. Bone-targeted AR overexpression results in a complex phenotype, predominantly in males, with increased trabecular bone mass (increased trabecular number but not thickness) in the setting of inhibition of resorption caused by reduced osteoclast activity. In addition, cortical formation is altered, with surface periosteal expansion but a lack of inner endosteal deposition. Inhibition of osteoclastic resorption might be responsible for altered trabecular morphology, consistent with reduced osteoclast activity and increased trabecular bone volume observed with androgen therapy in humans. The dramatic inhibition at the endosteal envelope could be responsible for the modest decrease in cortical bone area and changes in biomechanical properties observed. Thus, the bone phenotype observed in AR transgenic mice is consistent with many of the known effects of androgen treatment on the skeleton (Table 1). These results indicate that AR expressed in bone can be a direct mediator of androgen action to influence skeletal development and homeostasis.

Potential therapeutic options for bone
Testosterone treatment has been shown to be effective in stimulating bone turnover in boys with growth or pubertal delay [31•,47], and at ameliorating bone loss during aging in men with low testosterone levels [48•,49]. Nevertheless, androgen replacement in hypogonadal men remains a controversial issue [50] even though low testosterone is associated with increased mortality risk in geriatric patients [51]. It appears that androgens in a form that cannot be 5α-reduced might be preferable to satisfy concerns over prostate hypertrophy [40], as it appears that treatment with testosterone can be as effective as DHT with respect to preservation of bone mass [52•,53]. Testosterone may be effective at reducing bone formation in women with anorexia nervosa [54]. Furthermore, administration of androgenic anabolic steroids to stimulate bone mass shows promise in the treatment of severely burned children that normally develop growth inhibition or arrest [55•], and possibly in wasting diseases associated with androgen deficiency and reduced bone mass, such as HIV [56]. Anabolic steroids, however, might not be

effective in all settings [57]. Finally, selective androgen receptor modulators, analogous to selective estrogen receptor modulators used to influence estrogen receptor signaling, are being developed to protect bone and muscle but not stimulate prostate growth [58]. Although no selective androgen receptor modulators have been clinically approved, data indicate a bone protective effect in animals [59,60] and they may thus provide a new therapeutic option in the near future.

Conclusions
Androgens are important in the maintenance of a healthy skeleton, and have been shown to stimulate bone formation (Table 1). Androgens influence skeletal modeling and remolding by multiple mechanisms through effects on osteoblasts and osteoclasts and an influence on the differentiation of pluripotent stem cells toward the osteoblast lineage. The specific effects of androgen on bone cells are mediated directly through an AR signaling pathway, but there are also indirect contributions to overall skeletal health through aromatization and ER signaling. Androgens therefore provide promise for treatment in settings of low bone mass.

Update
As noted, androgens increase muscle mass and have thus been postulated to influence bone density through skeletal loading. Although it has been shown that appendicular skeletal muscle mass is positively correlated with bone mineral content and areal BMD in men [61•], controversy remains regarding the influence of fat mass versus muscle mass on bone formation in both sexes during aging and at specific sites [62•].

Interestingly, recent data demonstrate both direct inhibition of osteoclast formation and resorption by androgen treatment and an indirect effect through increased formation of osteoprotegerin via osteoblasts. In contrast, estrogen treatment had no direct effect on osteoclasts, whereas a similar indirect stimulation of osteoprotegerin was noted in osteoblasts [63••].

Additional support for the notion that androgens increase, whereas estrogens reduce, cortical bone size through opposing effects at the periosteal surface is reported in a recent cross-sectional study of 1068 young men [64•]. Notably, the apparent free testosterone concentration was found to be a positive predictor of cortical cross-sectional area and periosteal circumference, whereas free estradiol was an independent negative predictor of cortical bone size.

Using magnetic resonance microimaging to characterize trabecular architecture *in vivo*, Benito *et al.* [65••] show in a small sample of hypogonadal men that testosterone replacement results in improved trabecular connectivity, including an increase in trabecular thickness.

Acknowledgements

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Osteoblast and osteocyte apoptosis associated with androgen action in bone: Requirement of increased Bax/Bcl-2 ratio ☆

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Abstract

Both the number and the activity of osteoblasts are critical for normal bone growth and maintenance. Although a potential role for estrogen in protection of bone mass through inhibition of osteoblast apoptosis has been proposed, a function for androgen is much less clear. The aim of this study was to establish a direct role for androgen to influence osteoblast apoptosis both in vitro and in vivo. AR-MC3T3-E1 cells, with androgen receptor (AR) overexpression controlled by the type I collagen promoter, were treated with the non-aromatizable androgen 5α-dihydrotestosterone (DHT). Apoptosis was assessed by three different techniques including DNA fragmentation, caspase-3 activation, and changes in mitochondrial membrane potential. Transactivation of AR by DHT enhanced apoptosis while 17β-estradiol (E_2) treatment reduced apoptosis in both proliferating preosteoblasts and mature osteocyte-like cells. To explore mechanism, the apoptosis regulators Bcl-2 (antiapoptotic) and Bax (proapoptotic) were evaluated. Western analysis revealed that DHT decreased Bcl-2 resulting in a significantly increased Bax/Bcl-2 ratio. Regulation of Bcl-2 was post-transcriptional since *bcl-2* mRNA levels were unaffected by DHT treatment. Furthermore, ubiquitination of Bcl-2 was increased and serine phosphorylation was reduced, consistent with inhibition of MAP kinase signaling by DHT. Increased Bax/Bcl-2 ratio was essential since either Bcl-2 overexpression or Bax downregulation by RNA interference (RNAi) partially abrogated or reversed DHT-enhanced osteoblastic apoptosis. In order to establish physiologic significance in vivo, AR-transgenic mice with AR overexpression in the osteoblast lineage and thus enhanced androgen sensitivity were characterized. In male AR-transgenic mice, increased osteoblast apoptosis was observed in vivo even in association with new bone formation. Thus, although estrogen can be antiapoptotic, androgen stimulates osteoblast and osteocyte apoptosis through an increased Bax/Bcl-2 ratio even in anabolic settings. These results identify a new mechanism for androgen regulation of osteoblast activity distinct from estrogen, and suggest that enhanced apoptosis can be associated with anabolic stimulation of new bone growth. Androgens thus play a distinct role in skeletal homeostasis.
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Keywords: Osteoblast; Apoptosis; Androgen receptor; Androgen; Estrogen

Introduction

The skeleton is an important target organ for androgen action, distinct from estrogenic effects. Androgen receptors (AR) are expressed in osteoblasts, the cells responsible for osteogenesis. AR levels are increased in osteoblasts after androgen exposure [56,58] and as cells differentiate into a mature osteocytic phenotype [55]. The importance of AR signaling to influence skeletal homeostasis has been clearly demonstrated in both human [32] and mouse [51] models with androgen insensitivity, and in global AR null models [42,62]. Characterization of AR-transgenic mice with skeletally targeted AR overexpression has demonstrated proof of principle that direct androgen signaling in the osteoblast is an important mediator of androgen action in bone [59]. While the association of androgen with skeletal health and normal bone homeostasis is well established (for review, see [52]), the physiologic responses and molecular pathways influenced by androgen in

bone remain poorly characterized. The influence of estrogen on bone cell life span is an area of active investigation since it may be important in maintenance of bone mass, and play a role in the development of osteoporosis [48]. However, the effect of androgen is not well understood and is complicated by the fact that testosterone is the substrate for estradiol synthesis through aromatase activity. Thus, a controversy exists surrounding androgen's specific role(s) in bone homeostasis.

Androgen has been shown to influence bone cells in a complex fashion. For example, the effect of androgen on osteoblast proliferation has been shown to be biphasic in nature, with enhancement following short or transient treatment but significant inhibition following longer treatment (see [57]). Continuous treatment with the non-aromatizable androgen 5α-dihydrotestosterone (DHT) in proliferating preosteoblasts also resulted in decreased cell viability [57]. Reduced viability was associated with overall reduction in mitogen-activated (MAP) kinase signaling and with inhibition of *elk-1* gene expression, protein abundance, and extent of phosphorylation. Importantly, inhibition of MAP kinase signaling was observed both in normal primary rat calvarial cultures and in a model of enhanced androgen responsiveness, AR-MC3T3 cells. This result contrasts with stimulation of MAP kinase signaling and AP-1 transactivation observed with brief androgen exposure, that may be mediated through non-genomic mechanisms [24,28,63]. Since enhanced apoptosis is frequently associated with inhibition of MAP kinase signaling, the aim of the present study was to define the effects of androgen administration on osteoblast apoptosis in vitro and in vivo.

Apoptosis, or programmed cell death, is an essential process that is important for maintaining tissue homeostasis under normal conditions, in response to environmental insults and plays a vital role during embryogenesis that includes shaping of developing tissues [1]. In bone, apoptosis is important during embryonic limb development, skeletal maturation and modeling, adult bone turnover by remodeling, and during fracturing healing and regeneration [19]. One of the best characterized mechanisms to induce cell death is the release of proapoptotic proteins from the mitochondria into the cytosol (for a recent review, see [4]). Members of the Bcl-2 family, including Bcl-2 and Bax, are central regulators of apoptosis that promote (Bax) or inhibit (Bcl-2) cell death. Although a competition between Bax and Bcl-2 exists, each is able to regulate apoptosis independently [26]; thus, the ratio of Bax/Bcl-2 is an important indicator of apoptosis.

Previous reports indicate that Bcl-2 can be phosphorylated, and that phosphorylation of Bcl-2 is closely associated with regulation of apoptosis [8,14]. For example, positive regulation of Bcl-2 levels through MAP-kinase-mediated phosphorylation has been demonstrated, with activation of p44/42 MAP kinase inhibiting both downregulation of Bcl-2 protein and subsequent apoptosis [41]. The converse has also been observed, with inhibition of MAP kinase signaling shown to reduce Bcl-2 phosphorylation at consensus MAP kinase sites, leading to enhanced ubiquitination and ensuing Bcl-2 degradation in proteasomes [5,9]. Androgen treatment has been reported to reduce Bcl-2 levels [6,20] and enhance apoptosis [54,64] in

some tissues. Although we have demonstrated androgen-mediated reductions in MAP kinase signaling with chronic treatment [57], the impact of androgen treatment on levels of antiapoptotic Bcl-2 in osteoblasts has not been previously characterized.

Most analyses regarding effects of sex steroids on osteoblast apoptosis indicate that estrogen may be important in inhibiting apoptosis to increase osteoblast life span [39]. Few studies have directly analyzed the specific effects of androgens, however, leading to controversy regarding the nature of androgen signaling. In addition, it has been postulated that AR and estrogen receptor (ER) can activate an antiapoptotic non-genomic signaling pathway in osteoblasts with the same effectiveness regardless of whether the actual steroid ligand is an androgen or an estrogen [27]. Recent data, however, suggests that distinct, genomic signaling may be the more significant regulator in vivo [36,44,50]. In this study, the specific effect of androgen on osteoblast apoptosis through AR transactivation was examined in vitro with AR-MC3T3 cultures and in vivo in AR-transgenic mice. Interestingly, one facet of the low turnover bone phenotype observed in young male AR-transgenic mice is an anabolic response to androgen signaling [59], seen with an enhanced bone formation rate at the periosteum of long bones and with calvarial thickening. The effect of androgen on osteoblast apoptosis in an anabolic setting has not previously been examined. To gain further insights into the specific effect of androgen vs. estrogen on osteoblast apoptosis, we employed two novel models of enhanced androgen responsiveness; clonal col3.6 AR-MC3T3 cells (hereafter referred to as AR-MC3T3) stably transfected with full-length AR under control of 3.6 kb of the type I collagen promoter, and male col3.6 AR-transgenic mice (hereafter referred to as AR-transgenic mice). Results indicate dichotomous regulation of osteoblast apoptosis by androgen vs. estrogen, with chronic administration of androgen stimulating but estrogen inhibiting apoptosis.

Materials and methods

Reagents

All the media, buffers, supplements, and reagents for cell culture were obtained from GIBCO BRL-Life Technologies (Grand Island, NY) or Sigma Chemical Co. (St. Louis, MO). Steroid hormones and other reagents were obtained from Sigma Chemical Co. The active metabolite hydroxyflutamide (α,α,α-trifluoro-2-methyl-49-nitro-*m*-lactotoluidide, SCH 16423) was kindly provided by Schering-Plough Corp. (Madison, NJ). Hydroxyflutamide, an androgen receptor antagonist, was added to the cultures 30 min before hormone addition.

Cell culture

Cells stably transfected with AR (AR-MC3T3) or β-galactosidase (βgal-MC3T3) under the control of the rat 3.6 kb α1(I)-collagen promoter were created as previously described [57]. Cultures were maintained in minimal essential media (MEM) with 2.38 g/L HEPES and 2.2 g/L NaHCO₃ buffer, supplemented with 5% calf serum (CS). These cells were maintained throughout the study in the presence of G418 geneticin sulfate at 500 μg/ml. Cultures were treated with steroids in 5% charcoal-stripped CS. For appropriate osteoblast differentiation, cultures were switched at confluence to media containing 50 μg/ml ascorbic acid and 10 mM β glycerophosphate. Osteocytic cultures were

derived from cells grown 20–30 days (see [31,55,61]). Steroids were dissolved as stocks in ethanol and used at concentrations from 10^{-12} M to 10^{-8} M. The final ethanol concentration in the media was no higher than 0.1%.

Transient transfection, luciferase reporter assay

The potential of E_2 to stimulate ER transcriptional activation was determined using the E_2 response element (ERE) basal promoter-luciferase (4ERE-TATA-luciferase; ERE-luc) reporter construct containing 4 EREs linked to a minimal promoter, kindly provided by Dr. David Shapiro. ERE-luc was used to confirm that overexpression of AR did not influence transactivation of other steroid hormone receptors. AR-MC3T3 cells were grown in 6-well plates for 24 h before transfection. AR-MC3T3 cells were transfected with FuGENE 6 Transfection Reagent (Roche, Indianapolis, IN) using 1.5 μg of ERE-luc and 0.5 μg βgal expression vector for normalization in each well. Cultures were treated with vehicle or 10^{-12}–10^{-8} M E_2, with combined treatment with E_2 and 10^{-8} M DHT, or with DHT alone for 54 h. Cells were harvested and extracted in 300 μl reporter lysis buffer (Promega Corp, Madison WI). Luciferase and βgal activities were analyzed as previously reported [57]. The data shown represent the mean ± SEM from triplicate samples, performed in independent transfections two to three times.

Western blot analysis and immunoprecipitation

Protein was extracted from proliferating cell cultures as previously reported [55]. Polyclonal rabbit antibody recognizing AR (PA1-111A, Affinity Bioreagents Inc.) was used at 4 μg/ml. Both Bcl-2 and anti-mouse Bax polyclonal antibody (Δ21) specific for all 1–171 amino acids but the carboxy-terminal 21 amino acids (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were diluted at 1:200. As a loading control for total protein, Western blot analysis was performed with an anti-α-tubulin antibody at 1:1000 (Sigma, Saint Louis, MO). Membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody (Bio-Rad Laboratories, Richmond, CA) at 1:2000. Bound antibodies were visualized by ECL (Amersham Pharmacia Biotech, Piscataway, NJ) on a Kodak X-AR5 autoradiographic film.

For the detection of phosphorylated or ubiquitinated forms of Bcl-2, AR-MC3T3 cells were treated with 10^{-8} M of DHT for 72 h. To stimulate extracellular signal regulated kinase (ERK) activity, cultures were first starved in serum-free medium for 1 h with a subsequent addition of 5% charcoal stripped serum for 2 h. Equal amounts of protein were immunoprecipitated with anti-Bcl-2 antibody. Immunocomplexes were collected with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Inc.), resolved by 12% SDS-PAGE, and transferred onto Immobilon-P PVDF membrane. Western blot analysis after immunoprecipitation was carried out with either antiphosphoserine-specific antibody at 0.1 μg/ml (clone 4A9; Alexis Biochemicals, San Diego, CA), anti-Bcl-2 antibody as described above, or antiubiquitin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:300 dilution. Western blot analysis of extracts from AR-MC3T3 cells 48 h following mock transfection or transfection with the Bcl-2 expression plasmid or Bax siRNA was also performed. Quantitative analysis of the proteins was performed by volume densitometry using Optiquant Software (PerkinElmer Life and Analytical Sciences, Inc, Boston, MA) after scanning of the film (ScanMaker 9800 XL, Microtek, Carson, CA) in the linear range. Apoptosis data were expressed as the Bax/Bcl-2 ratio. Immunoprecipitation data are presented as the protein to α-tubulin ratio to correct for variations in protein loading, and then normalized to control values for comparison between treatments. The data shown represent the mean ± SEM.

AR quantification

Both AR-MC3T3 and control βgal-MC3T3 stable cultures were grown for 5 to 25 days, and AR binding analysis was performed as previously described [57]. ARs were quantitated by titration analysis using [^{3}H]methyltrienolone ([^{3}H]R1881) (New England Nuclear, specific activity 70–87 Ci/mM). Specific [^{3}H]R1881 binding capacity was estimated by one site binding with non-linear regression and Scatchard plot analysis using Prism v4 software (GraphPad Software, Inc, San Diego, CA). The high affinity (K_d = 1.0–5.0 × 10^{-10} M), specific binding of [^{3}H]R1881 was expressed in fmol/mg DNA and sites/cell.

The sensitivity for the detection of specific androgen binding using these methods was 5 fmol/ml.

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and primer sequence

Confirmation of endogenous AR and colAR transcript expression was performed using qRT-PCR with the iCycler IQ Real-Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA) using a one-step QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA) on DNase-treated total RNA as previously described [59]. AR transgene mRNA was amplified with 5′-GCATGAGCCGAAGCTAAC-3′ and 5′-GAACGCTCCTCGATAGGTCTTG-3′. These primers were designed to specifically amplify the transgene using sites in the collagen untranslated region and part of the AR sequence. Endogenous mouse AR mRNA was amplified with 5′-GGAATTCGGTGGAAGCTACA-3′ and 5′-CCGGGAGGTGCTATGT-3′. To determine the mRNA levels of *bcl-2* and *bax* after treatment with DHT or E_2, murine *bcl-2* mRNA was amplified with 5′-TTCGCAGAGATGTCCAGTCA-3′ and 5′-CACCCCATCCCTGAA-GAGTT-3′; murine *bax* mRNA was amplified with 5′-CCAAGAAGCTGAGC-GAGTGT-3′ and 5′-TGTCCACGTCAGCAATCATC-3′. Relative expression of the RT-PCR product was determined using the comparative $\Delta\Delta C_t$ method after normalizing expression with either 18S rRNA or fluorescence to the specific RNA binding dye RiboGreen (Molecular Probes, Eugene, OR, USA) as previously described [15]. Real-time qRT-PCR efficiency was determined for each primer set using a 5-fold dilution series of total RNA and did not differ significantly from 100%. Following PCR, specificity of the PCR reaction was confirmed with melt curve analysis. Amplicons were also sequenced for confirmation. Data are presented as mean ± SEM.

Assessments of apoptosis

AR-MC3T3 cells were plated in 6-well dishes and grown for either 5 days (proliferating) or 29 days (osteocyte-like) in the continuous presence of either vehicle or 10^{-8} M DHT or 10^{-8} M E_2. Apoptosis was induced either with 50 μM etoposide (ETOP) for 18 h before harvest or by serum starvation for 48 h, replacing serum with 0.1% bovine serum albumin. Three independent methods were employed for analysis of apoptosis after these treatments.

Cytoplasmic nucleosome enrichment

Quantitative in vitro determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) in combined attached and detached cell pools of AR-MC3T3 treated as described above used the Cell death ELISA^{Plus} (Roche Diagnostics, Indianapolis, IN) kit. Absorbance was measured at 405 nm, and data are expressed as the nucleosome enrichment factor = $A_{treated}/A_{untreated}$.

Caspase-3/CPP-32 activity

Apoptosis-induced activation of caspase-3 activity in treated AR-MC3T3 cultures was assessed by measuring colorimetric substrate DEVD-*p*NA cleavage (BioVision, Inc.) according to manufacturer's suggestions in a homogenate containing 50 μg protein.

Mitochondrial membrane potential

Loss in mitochondrial membrane potential ($\Delta\Psi_{mito}$) in treated AR-MC3T3 cells after induction of apoptosis was detected using ApopAlert Mitochondrial Membrane Sensor kit (BD Biosciences Clontech, Palo Alto, CA). AR-MC3T3 cultures were rinsed with serum-free media, stained with BD MitoSensor Dye at 37°C for 20 min, and analyzed by fluorescence microscopy.

In vivo histological analysis

AR-transgenic mice with AR overexpression in the osteoblast lineage were employed for in vivo histological analysis. AR-transgenic mice, previously generated and characterized [59], were healthy and transmitted the transgene at the expected frequency. For experiments, hemizygous littermates were obtained by mating transgenic founders with B6D2F1 wild-type mice of both genders. Calvaria were isolated from AR-transgenic mice and littermate controls at 2

months and 6 months old, fixed in 4% paraformaldehyde, decalcified in Immunocal (Decal Corp., Tallman, NY), and then processed for paraffin embedding as previously described [59]. 5–6 μm sections were cut and stained with hematoxylin and eosin (H&E). New bone growth was localized in calvarial cross sections using van Geison staining to identify collagen synthesis that has formed within 4 days of isolation [17]. Apoptosis-induced DNA fragmentation in bone sections was assessed by means of Terminal-dUTP-Transferase-Nick-End Labeling (TUNEL) staining using TdT-FragEL DNA fragmentation detection kit (Oncogene research products, Boston, MA) following manufacturer's recommendations. Detection was performed using a streptavidin–horseradish peroxidase conjugate and 3,3′-diaminobenzidine as a color substrate. Sections were counterstained with methyl green, mounted, and viewed under a microscope. Apoptotic cells are identified with condensed nuclei and dyed brown. Positive and negative controls were always included. Positive control sections were treated with 1 μg/μl DNase I in TBS with 1 mMgSO$_4$ for 20 min at room temperature following proteinase K treatment. Negative control sections were incubated without TdT.

Transient transfection, overexpression, and RNA interference (RNAi) analysis

Experiments were performed to alter Bax/Bcl-2 ratio by transient transfections. Bcl-2 overexpression was achieved using pCMV-Bcl-2 expression construct (kindly provided by Dr. Stanley Korsmeyer, Harvard Medical School). AR-MC3T3 cells were plated onto 6-well dish and transfected with 2 μg of DNA per well using FuGENE 6 transfection (Roche, Basel, Switzerland) reagent at a 6:1 ratio (FuGene6: μg DNA). To inhibit *bax* gene expression, an oligonucleotide-based technique with double-stranded short interfering RNAs (siRNA) validated for *bax* was used, following manufacturer's instructions. Bax siRNA was introduced via transient transfection with SureSilencing Mouse Bax siRNA Kit (SuperArray Bioscience Corp., Frederick, MD) and Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA). Transfections included negative control siRNA populations. AR-MC3T3 cells were transfected with indicated sequences (pCMV-Bcl-2 or Bax siRNA). Cultures were exposed to 10^{-8} M DHT for 30 h followed by 18 h-treatment with 50 μM etoposide and then isolated at day 5. Apoptosis was evaluated using the Cell Death oligonucleosome assay in transfected and mock-transfected cultures. The data are expressed as the mean ± SEM. Confirmation of alteration of protein levels of Bcl-2 and Bax was determined with Western blotting (see Fig. 6).

Statistical analysis

All data were analyzed using Prism v4.0 software (GraphPad Software, Inc.; San Diego, CA). Values from duplicate or triplicate wells from two to three experiments were used for statistical analysis with Student's *t* test or one-way ANOVA followed by post hoc analysis with Newman–Keuls Multiple Comparison Test. Differences of $P < 0.05$ were considered statistically significant. Results are presented as the mean ± SEM.

Results

Model of enhanced androgen responsiveness

AR levels are low as osteoblasts proliferate but increase during differentiation in vitro to reach maximal levels in mature osteocytic-like cells [55]. In order to enhance androgen responsiveness, particularly in early proliferating osteoblasts, we have employed cultures of the immortalized mouse calvarial osteoblastic cell line MC3T3-E1 stably transfected with an AR expression construct under the control of 3.6 kb of the rat α1(I)-collagen promoter (for details, see reference [57]). AR-MC3T3 cells were used as stable pools and grown under selection with G418.

To determine total AR levels throughout in vitro differentiation, both proliferating and osteocytic AR-MC3T3 cultures were characterized with AR binding and Scatchard plot analyses (Table 1). As can be seen, there is an approximately 3-fold elevation in AR binding between control βgal-MC3T3 vs. AR-MC3T3 cultures in proliferating cultures at day 5. In mature osteoblastic/osteocytic cultures at day 25, there is also approximately 3-fold elevation in AR levels compared to control cultures. In addition, AR levels nearly doubled during differentiation in AR-MC3T3 cultures, with a less robust increase in control cultures. Analysis by two-way ANOVA demonstrated that both cell line and time in culture significantly influenced AR levels. AR protein abundance was also determined by Western analysis in whole cell lysates isolated during proliferation (day 5) and at a more mature osteoblast stage (day 20). As shown in Fig. 1A, total AR levels (from both endogenous AR and colAR transgene expression) are elevated in mature cultures. We next analyzed expression of the colAR transgene by qRT-PCR analysis using total RNA isolated from AR-MC3T3 cultures with primers specific for the colAR transgene or for endogenous AR (Fig. 1B). AR-transgene expression was elevated approximately 1.7-fold relative to the endogenous AR gene.

In order to evaluate whether overexpression of AR in these cultures alters steroid receptor signaling in a generalized non-specific fashion, we characterized transactivation mediated by a distinct steroid receptor, i.e., estrogen transactivation of ERs in AR-MC3T3 cultures. ER activity was evaluated in transient transfections using the ERE reporter construct ERE-luc. Cells were treated for 54 h with E_2 in a dose response (10^{-12} M–10^{-8} M) in the presence or absence of 10^{-8} M DHT. AR-MC3T3 cultures demonstrated a dose-dependent stimulation in ER transactivation with approximately 1.6-fold elevation with 10^{-8} M E_2. Importantly, E_2 signaling was not altered during coincident AR transactivation by cotreatment with combined 10^{-8} M E_2 and DHT, nor was DHT alone sufficient to activate ERE-dependent ER transactivation (Fig. 1C).

Table 1
Analysis of AR levels with differentiation in AR-MC3T3 cultures

Tissue/cells	$K_d \times 10^{-10}$ M	Bmax (fmol/mg DNA)	Capacity (sites/cell)
d5 βgal-MC3T3	1.9 ± 0.45	644 ± 138	2323 ± 496
d5 AR-MC3T3	2.2 ± 0.05	1491 ± 16	5383 ± 60
d15 βgal-MC3T3	1.5 ± 0.03	991 ± 160	3576 ± 576
d15 AR-MC3T3	2.9 ± 0.60	1446 ± 86	5219 ± 311
d25 βgal-MC3T3	1.8 ± 0.20	1308 ± 469	4720 ± 1691
d25 AR-MC3T3	2.6 ± 0.20	2479 ± 405	8947 ± 1461
Ventral prostate	5.8 ± 0.06	1351 ± 85	4877 ± 308
LNCaP	7.7 ± 1.57	3971 ± 598	14,335 ± 2157

Androgen receptor levels were quantitated by titration analysis using [³H] methyltrienolone ([³H]R1881). The DNA content was quantitated and specific [³H]R1881 binding capacity was estimated by one site binding with non-linear regression and Scatchard plot analysis. The specific binding of [³H]R1881 was expressed in fmol/mg DNA and sites/cell. Analysis by two-way ANOVA demonstrated that both cell line and time in culture significantly influenced AR levels.
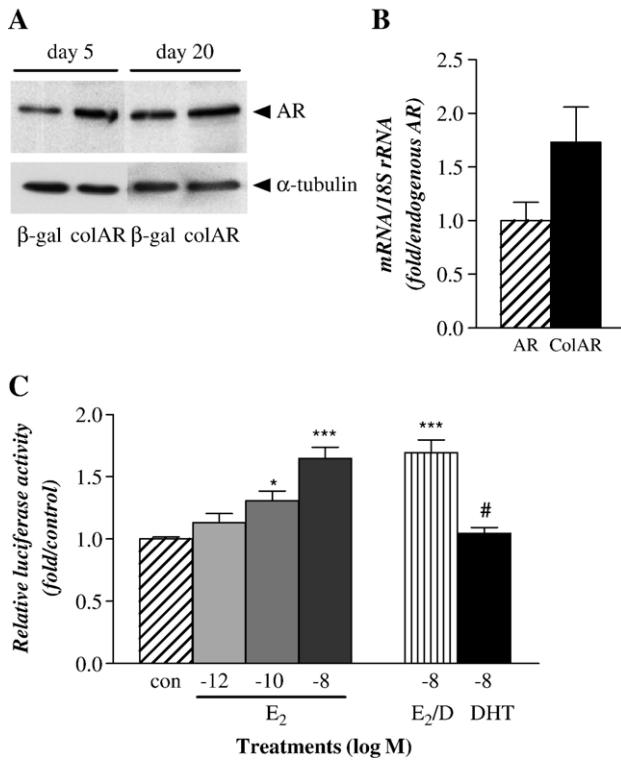
Fig. 1. Characterization of AR levels in AR-MC3T3 cultures during osteoblast differentiation. AR levels in AR-MC3T3 cultures during proliferation (day 5) and in mature osteoblasts/osteocytes (day 20) were assessed by Western analysis. (A) AR protein levels in stably transfected AR-MC3T3 and control βgal-MC3T3 cells. Both AR-MC3T3 and control βgal-MC3T3 cells were grown for the indicated time in culture, and AR abundance was determined in whole cell lysates with Western blot analysis using polyclonal rabbit antibodies recognizing AR (PA1-111A). The same membrane was reprobed with α-tubulin antibodies as a loading control. The increase in AR levels with differentiation is also seen by Scatchard plot analysis presented in Table 1. (B) Characterization of colAR transgene gene expression by qRT-PCR analysis. Primers specific for the colAR transgene and for endogenous AR were used to evaluate relative AR transgene expression using total RNA isolated from AR-MC3T3 cultures. (C) Lack of effect of AR overexpression on transactivation of other steroid receptors. ER function was evaluated in AR-MC3T3 cultures in transient transfections using the ERE reporter construct ERE-luc. Cells were treated for 54 h with E_2 at 10^{-12} M, 10^{-10} M, and 10^{-8} M in the presence or absence of 10^{-8} M DHT. The data are expressed as the means ± SEM ($n = 6$) from two independent experiments. Analysis by ANOVA revealed significant differences ($P < 0.0001$). Post hoc analysis using Newman–Keuls Multiple Comparison Test demonstrated a significant increase in reporter activity with E_2 treatment, but no effect of DHT alone or in the presence of E_2. *$P < 0.05$; ***$P < 0.001$ vs. control; #$P < 0.001$ vs. E_2 + DHT combined.

Characterization of osteoblast apoptosis: androgen and estrogen effects during proliferation

We have previously shown that continuous DHT treatment reduces osteoblast viability through reductions in MAP kinase signaling in both normal rat primary calvarial cells and in AR-MC3T3 cultures [57]. Since reductions in viability can be associated with enhanced apoptosis, we sought to determine whether DHT treatment also influenced osteoblast apoptosis. Three independent assays were employed to characterize apoptosis: nucleosome fragmentation, caspase-3 activity, and $\Delta \Psi_{mito}$ analysis. AR-MC3T3 cells were plated in 6-well dishes

and grown for 5 days in the continuous presence of either vehicle, 10^{-8} M DHT, or 10^{-8} M E_2. Apoptosis was induced by treatment with 50 μM etoposide for 18 h before harvest then assessed by oligonucleosome analysis (Fig. 2A) and with determination of caspase-3/CPP32 activity measured by substrate cleavage (Fig. 2B). DHT treatment significantly enhanced osteoblast apoptosis 1.5- to 2-fold, in the range described for well-characterized proapoptotic effects of glucocorticoid or TNFα (see [2,45]) and with BMP-2 administration (see [16]) in osteoblasts. Separate one-way ANOVAs for each treatment determined that both androgen and estrogen treatments were significantly different from control but with opposite effects: DHT treatment significantly enhanced while E_2 treatment inhibited apoptosis. Although not as robust presumably due to lower AR levels, DHT also stimulated apoptosis in control βgal-MC3T3 cultures (data not shown).

To extend these results, mitochondrial membrane permeability $\Delta \Psi_{mito}$ analysis was performed. AR-MC3T3 cells were rinsed with serum-free media, stained with MitoSensor Dye at 37°C for 20 min, and analyzed by fluorescence microscopy (Fig. 2C). Collapse of the electrochemical gradient across the mitochondrial membrane is an early indicator of the initiation of cellular apoptosis. MitoSensor (cationic dye) is taken up in the mitochondria where it forms red fluorescent aggregates in healthy cells. In apoptotic cells, the dye cannot aggregate because of altered $\Delta \Psi_{mito}$ and remains in a green fluorescent monomeric form in the cytoplasm. Modestly increased green/yellow staining with etoposide, enhanced with DHT treatment, is consistent with loss of mitochondrial membrane permeability and $\Delta \Psi$. In contrast, red stained cells with E_2 treatment suggests that cultures are relatively protected from etoposide-induced apoptosis. In control cultures without etoposide induction, DHT and E_2 demonstrated similar but even less robust effects.

To establish whether androgen-induced apoptosis was mediated by functional AR, AR-MC3T3 cultures were treated with DHT in the presence of the specific non-steroidal AR antagonist hydroxyflutamide (OHF). As shown in Fig. 2D, an ~2.2-fold increase in apoptosis is observed after 10^{-8} M DHT treatment for 5 days ($P < 0.01$). However, coincubation with 10^{-6} M OHF significantly abrogated the effect of DHT on apoptosis ($P < 0.05$). OHF alone appeared to have a slight agonist effect as has been noted previously (see [56]); however, the effect was not significantly different from control.

Characterization of osteoblast apoptosis: androgen effects during differentiation in mature osteoblast/osteocyte cultures

Since much of the analysis of the effects of estrogen on osteoblast apoptosis has focused on osteocytic cells, known to demonstrate enhanced apoptosis [31], we next determined whether the effect of either estrogen or androgen treatment on osteocytic cells in extended culture at day 29 was similar to that observed in proliferating osteoblasts. We have shown that both ERα and ERβ mRNA and protein expression in osteoblastic cells were similar or elevated at days 25 or 30 when compared
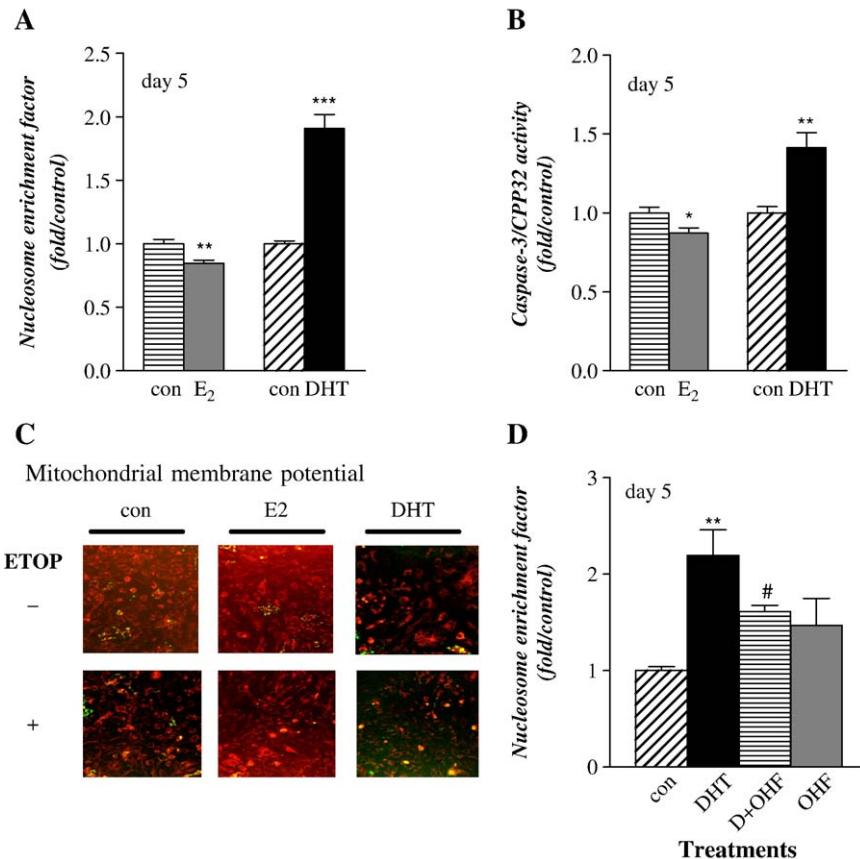
Fig. 2. Characterization of osteoblast apoptosis: results of androgen and estrogen treatment during proliferation. AR-MC3T3 cells were plated in 6-well dishes and grown for 5 days in the continuous presence of vehicle or 10^{-8} M DHT or 10^{-8} M E_2. Apoptosis was induced by treatment with 50 μM etoposide for 18 h before harvest. Apoptosis was characterized using three independent assays. (A) Cell Death oligonucleosome analysis. Apoptosis was characterized by analyzing DNA fragmentation with the Cell Death oligonucleosome assay. The data are presented as the nucleosome enrichment factor = $A_{treated}/A_{untreated}$, expressed as the means ± SEM (n = 9–12) from three separate studies. Differences were statistically significant by Student's t test. **P < 0.01; ***P < 0.0001 vs. control. (B) Caspase-3/CPP32 activity measured by substrate cleavage. Data are presented as mean ± SEM (n = 8). Experiments were carried out three times. *P < 0.05; **P < 0.01 vs. control. (C) Mitochondrial membrane potential ($\Delta \Psi_{mito}$) analysis. AR-MC3T3 cells were rinsed with serum-free media, stained with MitoSensor Dye at 37°C for 20 min, and analyzed by fluorescence microscopy. All results are representative of at least three independent experiments. (D) Characterization of osteoblast apoptosis: dependence on AR function. AR-MC3T3 cells were cultured as described with 10^{-8} M DHT treatment for 5 days with or without 10^{-6} M hydroxyflutamide (OHF), an androgen receptor antagonist. Apoptosis was evaluated by Cell Death oligonucleosome analysis. The data are expressed as the means ± SEM (n = 6) from two independent experiments. Differences were statistically significant by one-way ANOVA (P = 0.0033). Post hoc analysis with Newman–Keuls Multiple Comparison Test demonstrated that hydroxyflutamide significantly abrogates DHT-mediated apoptosis at day 5. **P < 0.01 (vs. control); #P < 0.05 (vs. DHT).

with day 5 [55], and that AR-MC3T3 osteocytic cultures have enhanced AR expression (Fig. 1 and Table 1). Osteocytic cultures were characterized after continuous DHT and E_2 treatment (both at 10^{-8} M) for 29 days (Fig. 3). Cultures were serum starved for the last 48 h in medium containing 0.1% bovine serum albumin. Apoptosis was induced in osteocytic cultures by serum starvation instead of etoposide administration since topoisomerase II activity (the target of etoposide treatment) is dramatically reduced in highly confluent cultures [47]. Quantitative analysis of apoptosis was determined using nucleosome fragmentation, caspase-3 activity, and mitochondrial membrane permeability as described in Fig. 2. Consistent with results in proliferating cultures, DHT enhanced but E_2 inhibited osteoblast apoptosis after continuous treatment in day 29 osteocytic cultures assessed by oligonucleosome analysis (Fig. 3A) and loss of mitochondrial membrane permeability (Fig. 3C). DHT treatment modestly increased caspase-3

activity but this result did not reach significance (Fig. 3B); however, E_2 did not suppress caspase-3 activity. These results may reflect the high levels of caspase-3 expression in osteocytic cultures in vitro [35].

DHT-mediated changes in key apoptotic regulators Bcl-2 and Bax

In order to characterize the mechanism underlying DHT-enhanced osteoblast apoptosis, we next determined the effect of androgen treatment on the important apoptotic regulators Bcl-2 and Bax. Western blot analysis was used to determine the levels of Bcl-2 and Bax protein in DHT- or E_2-treated AR-MC3T3 cells (Fig. 4A). After DHT treatment, we observed significant enhancement of the Bax/Bcl-2 ratio by nearly 1.3-fold (P < 0.05; Fig. 4B), which mostly reflected a decrease in Bcl-2 protein levels. E_2 treatment alone did not significantly alter the
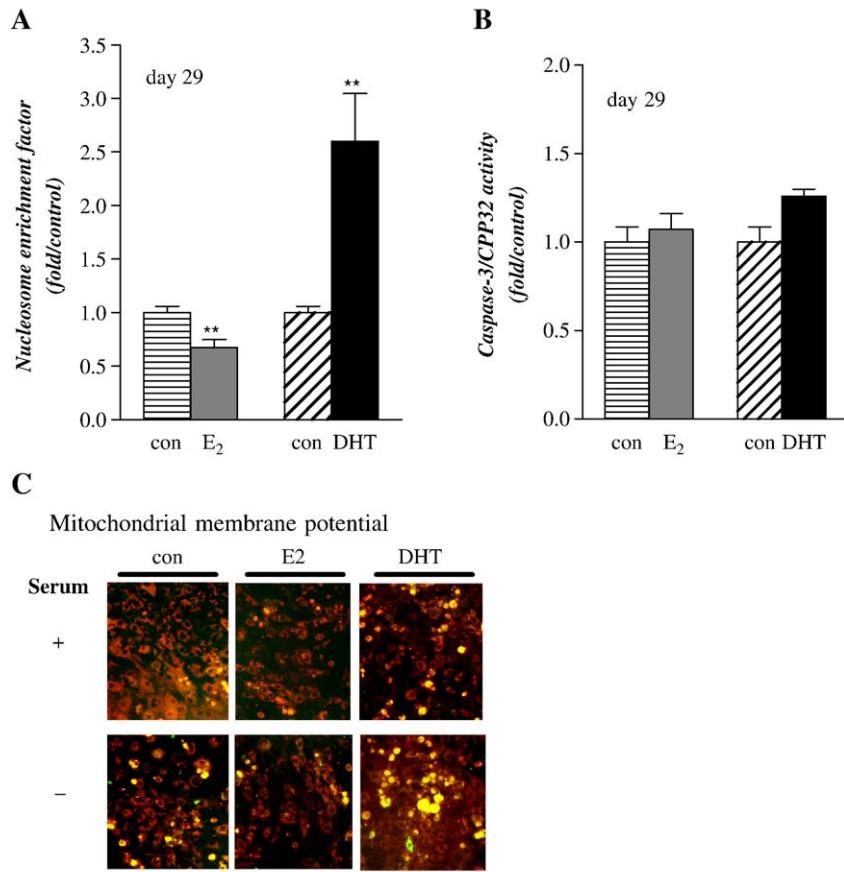
Fig. 3. Characterization of osteoblast apoptosis: androgen effects during differentiation in mature osteoblast/osteocyte cultures. Apoptosis was characterized in osteocyte-like cultures after continuous DHT and E_2 treatment (both at 10^{-8} M) for 29 days. Apoptosis was induced by serum starvation for 48 h before isolation, replaced with 0.1% BSA. (A) Analysis of apoptosis evaluating DNA fragmentation analysis. The data are expressed as the means ± SEM ($n = 6$) from two independent experiments. See Fig. 2 for methods. **$P < 0.01$ vs. control. (B) Caspase-3/CPP-32 activity measured by substrate cleavage. Lysates from day 29 control and hormone-treated cultures were analyzed. The data are expressed as the means ± SEM ($n = 6$) from two independent experiments. (C) Mitochondrial membrane potential ($\Delta \Psi_{mito}$) analysis. AR-MC3T3 cells were cultured with continuous DHT and E_2 treatment (both at 10^{-8} M) for 29 days, stained with MitoSensor Dye, and analyzed by fluorescence microscopy. All results are representative of at least three independent experiments.

Bax/Bcl-2 ratio, consistent with previous analysis in osteoblasts [12]. To better characterize the time course of changes in Bcl-2 protein, Western analysis was performed after 24, 48, 72, and 96 h of continuous 10^{-8} M DHT treatment (Fig. 4C). Quantitative analysis of Bcl-2 protein levels indicated by fold change showed that Bcl-2 levels were relatively unaffected for up to 48 h of DHT treatment, but then decreased by 72 h and 96 h of treatment to ~70% of control levels. This result is consistent with the time course observed for androgen-mediated changes in osteoblast viability [57].

We next investigated the mechanism by which androgen treatment stimulated the proapoptotic response by determining bcl-2 and bax steady-state mRNA levels with real-time qRT-PCR analysis. AR-MC3T3 cells were incubated continuously with either DHT or E_2 (both at 10^{-8} M) for 5 days and total RNA was isolated. Fig. 4D shows that bax mRNA levels were modestly elevated ~20% with DHT treatment, although this effect did not reach statistical significance. In contrast, bcl-2 mRNA levels were not different compared to those for control vehicle-treated cells. Given the reductions in Bcl-2 protein but no corresponding decrease in bcl-2 mRNA levels, these results

suggest that DHT regulation of Bcl-2 protein levels is post-transcriptional.

The androgen-mediated reduction of Bcl-2 protein is associated with decreased Bcl-2 serine phosphorylation and increased ubiquitination

MAP kinase activity has been shown to influence Bcl-2 protein degradation through changes in Bcl-2 phosphorylation [5,9]. Since we have previously demonstrated reduced MAP kinase signaling after chronic androgen treatment in osteoblastic cells [57], we sought to determine whether androgen treatment in osteoblasts also reduced Bcl-2 phosphorylation. AR-MC3T3 cells were treated with 10^{-8} M DHT for 3 days and whole cell extracts were prepared. Equal amounts of protein were analyzed for the presence of total and phosphorylated Bcl-2 after immunoprecipitation followed with Western blot analysis using either pan Bcl-2 or antiphosphoserine-specific antibody (clone 4A9) for detection (Fig. 5A, left panel). DHT treatment reduced Bcl-2 protein to ~80% of control levels, with an additional reduction in serine phosphorylation to ~70% of
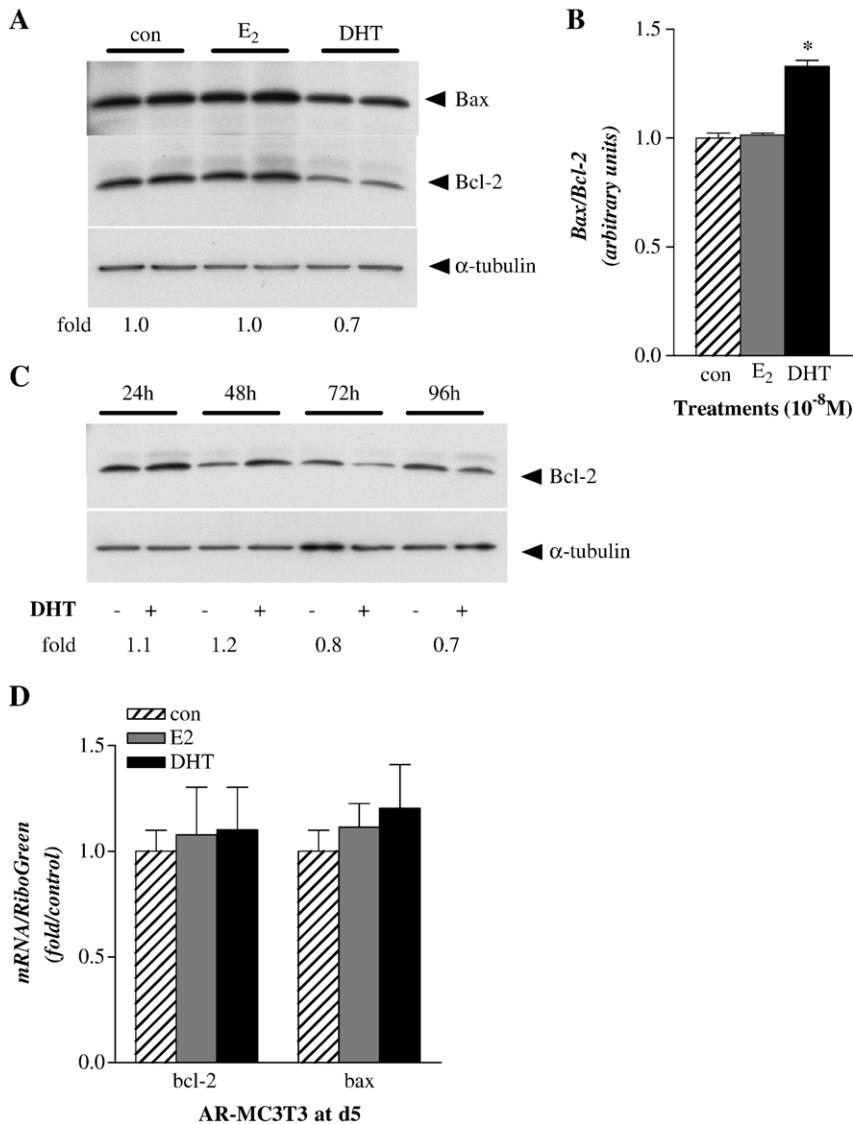
Fig. 4. DHT-mediated changes in key apoptotic regulators Bcl-2 and Bax. AR-MC3T3 cells were isolated at day 5 after continuous DHT or E_2 (10^{-8} M) treatment. (A) Western blot analysis of Bcl-2 and Bax levels. Total cell lysates from AR-MC3T3 treated with steroids for 5 days were used to evaluate Bcl-2 and Bax protein levels. Extract was analyzed by 15% SDS-PAGE, transferred onto an Immobilon-P PVDF membrane and probed using polyclonal rabbit antibodies recognizing Bcl-2 or Bax as described in Materials and methods. Fold difference between control and DHT treatment is indicated for Bcl-2 levels. (B) Quantitative analysis of Bax/Bcl-2 ratio. Quantification of immunoband intensities from Western blot analysis as shown in panel A was determined by densitometric scanning. Data were expressed as Bax/Bcl-2 ratio. Results are representative of at least three independent experiments. *$P < 0.05$ vs. control. (C) Time course of Bcl-2 protein levels after androgen treatment. AR-MC3T3 cultures were treated with 10^{-8} M DHT for 24, 48, 72, and 96 h, and Bcl-2 levels were characterized by Western blot analysis. Fold difference between control and DHT treatment is indicated for Bcl-2 levels. (D) Regulation of Bcl-2 is post-transcriptional. Levels of *bcl-2* and *bax* mRNA were analyzed by qRT-PCR analysis from total RNA isolated from control, E_2, or DHT-treated AR-MC3T3 cultures for 5 days. Levels of *bcl-2* were unaffected by hormone treatment, while the levels of *bax* were modestly increased by DHT. The data are expressed as the means ± SEM ($n = 4$) from two experiments.

normal levels (Fig. 5A, right panel). To provide further evidence that proteasomes were involved in Bcl-2 degradation after androgen treatment, AR-MC3T3 cells were treated with DHT and the effect on ubiquitination of Bcl-2 was determined. Total Bcl-2 was isolated from whole cell lysates by immunoprecipitation followed by Western blot analysis with antiubiquitin antibody. As shown in Fig. 5B, ubiquitin conjugates of Bcl-2 were modestly increased by DHT treatment, consistent with post-transcriptional regulation. Combined, these results suggest that targeting of Bcl-2 for degradation by proteasomes is associated with reduced serine phosphorylation of Bcl-2 protein.

The apoptotic effect of DHT requires an increase in Bax/Bcl-2 ratio

In order to determine whether apoptosis stimulated by androgen treatment requires an increase in Bax/Bcl-2 ratio, expression of either *bcl-2* or *bax* mRNA was altered by transiently transfection. To reverse the inhibition of Bcl-2 expression by androgen treatment, Bcl-2 was overexpressed using the pCMV-Bcl-2 expression construct. AR-MC3T3 cells were transfected with pCMV-Bcl-2 or mock-transfected, then treated with DHT for a total of 48 h (30 h followed by
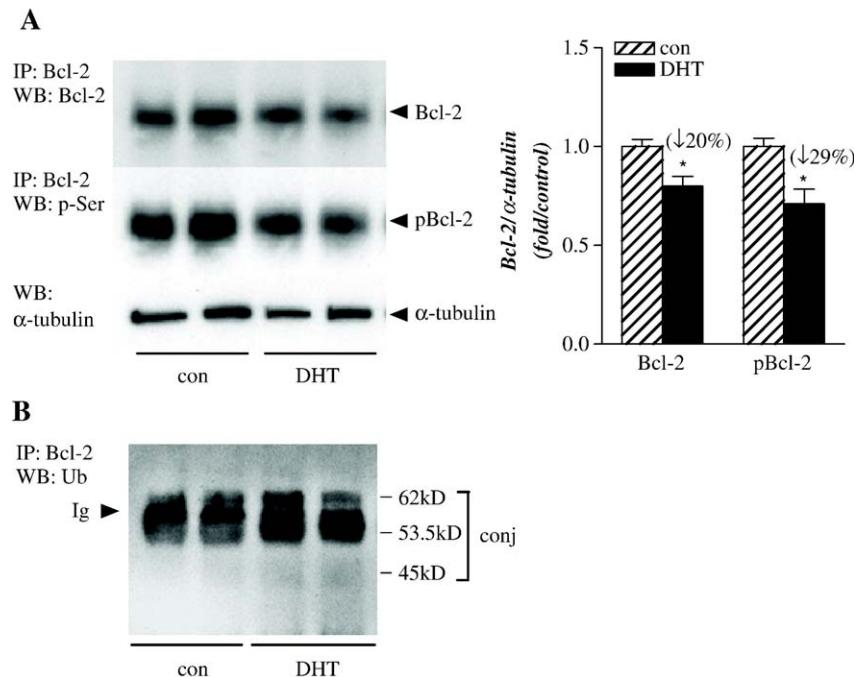
Fig. 5. The androgen-mediated reduction of Bcl-2 protein is associated with decreased Bcl-2 phosphorylation and proteasomal degradation. AR-MC3T3 cells were treated with 10^{-8} M DHT for 72 h and equal amounts of protein were immunoprecipitated with anti-Bcl-2 antibody. Immunocomplexes were resolved by 12% SDS-PAGE and transferred onto Immobilon-P PVDF membrane. (A) Androgen treatment reduces Bcl-2 protein and induces Bcl-2 dephosphorylation. Western blot (WB) analysis was carried out with either anti-Bcl-2 antibody or antiphosphoserine-specific antibody after immunoprecipitation (IP). Equal protein loading was confirmed with α-tubulin levels determined before immunoprecipitation by Western analysis from the same lysates. Quantification of immunoband intensities was determined by volumetric densitometric scanning (right panel). The data are expressed as the means ± SEM ($n = 4$) from two independent experiments. *$P < 0.05$ vs. control. (B) Androgen induces Bcl-2 ubiquitination. Ubiquitin conjugates of Bcl-2 were identified with Western blot analysis using antiubiquitin antibody after immunoprecipitation. conj., conjugates; Ig, the heavy chain of the immunoglobulin molecule.

50 μM etoposide for 18 h). Apoptosis was characterized at day 5 using mono- and oligonucleosome analysis as described in Fig. 2. In osteoblastic cultures with Bcl-2 overexpression (Fig. 6A), androgen-enhancement of apoptosis was completely reversed ($P < 0.001$), confirming the importance of reductions in Bcl-2 levels. Overexpression of Bcl-2 protein was confirmed by Western analysis (Fig. 6C, left panel).

We next employed RNAi to inhibit Bax protein using an oligonucleotide-based technique with double-stranded siRNA validated for *bax*. AR-MC3T3 cells were transfected with siRNA Bax-specific oligonucleotides 24 h prior to the exposure of cells to DHT for 30 h, followed by 50μM etoposide for 18 h before isolation at day 5. As shown in Fig. 6B, DHT-mediated increases in osteoblast apoptosis were significantly abrogated with Bax inhibition ($P < 0.001$). Western analysis of Bax protein levels in androgen treated AR-MC3T3 cells expressing Bax siRNA (Fig. 6C, right panel) confirmed that siRNA treatment resulted in a reduction in Bax protein expression to 60% of normal levels, with no effect after transfection with control siRNA oligonucleotides. A second source of *bax* siRNA oligonucleotides also abrogated DHT-mediated increases in osteoblast apoptosis (data not shown). These data suggest that an increase in the Bax/Bcl-2 ratio is important for androgen-mediated apoptosis, and is consistent with reports that Bcl-2 and Bax can function independently to regulate apoptosis [26].

In vivo stimulation of osteoblast apoptosis in AR-transgenic mice

In order to assess the physiological relevance of these observations, we characterized apoptosis in skeletally targeted AR-transgenic mice (for a description of the phenotype observed in these animals, please refer to reference [59]). AR-transgenic mice were created with the same full-length AR expression construct used for the AR-MC3T3 cells, demonstrate AR overexpression throughout the osteoblast lineage, and represent a model of enhanced responsiveness to circulating androgen without systemic hormone administration. An anabolic response to androgen in males was observed in H&E stained sections from calvaria isolated from male AR-transgenic mice vs. littermate controls at 2 months (Fig. 7A, left panels) and 6 months (Fig. 7A, right panels), and was not observed in females as previously reported [59]. Localization of new bone growth was characterized in calvarial cross sections using van Geison staining, where recently formed collagen is identified by a characteristic blue color [17]. As can be seen in Fig. 7B, new bone growth was present in calvaria as a result of AR-transactivation in post-pubertal (i.e., 2 month old; left panel) and adult (i.e., 6 month old; right panel) male AR-transgenic mice. There may be increased cell density in the woven bone present in the AR-transgenic male calvaria, consistent with the notion that woven bone has been estimated to contain 4 to 8 times more osteocytes than lamellar bone [18].

A

Bcl-2

B

Bax

C

Western blot analysis
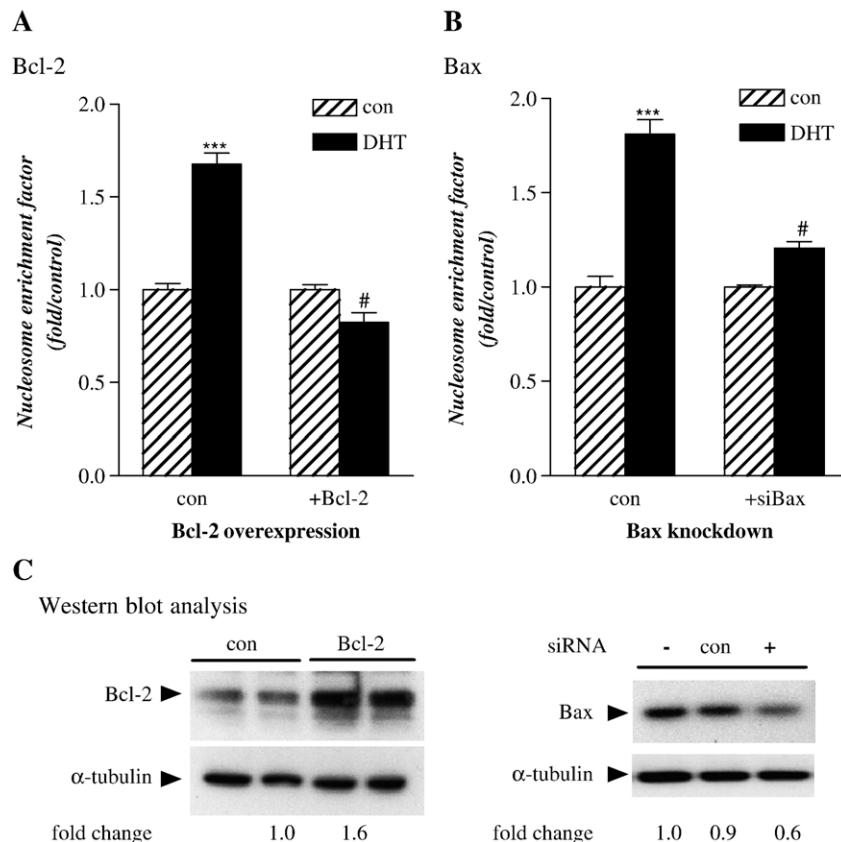
Fig. 6. The proapoptotic effect of DHT requires a decline in Bcl-2/Bax ratio. Bcl-2 or Bax levels were altered in AR-MC3T3 cultures after transient transfection. Cultures were transfected 1 day after plating and exposed to 10^{-8} M DHT for 48 h. Apoptosis was induced by treatment with 50 μM etoposide for 18 h before isolation at day 5.(A) Effect of Bcl-2 overexpression. Bcl-2 mRNA overexpression was achieved by transfection with expression construct pCMV-Bcl-2. Apoptosis was evaluated using the Cell Death oligonucleosome assay in transfected and mock-transfected cultures. The data are expressed as the mean ± SEM ($n = 6$) from two experiments. Differences were statistically significant by one-way ANOVA ($P < 0.0001$). Post hoc analysis with Newman–Keuls Multiple Comparison Test demonstrated that overexpression of Bcl-2 completely reverses DHT-mediated apoptosis at day 5. ***$P < 0.001$ (vs. control); #$P < 0.001$ (vs. DHT control). (B) Effect of Bax knockdown with double-stranded siRNA treatment. Cultures were transfected with control siRNA (con) or Bax siRNA (double-stranded Bax siRNA) using the validated SureSilencing Mouse Bax siRNA kit as described in Materials and methods or were mock-transfected. Apoptosis was evaluated using the Cell Death oligonucleosome assay in transfected and control cultures. The data are expressed as the mean ± SEM ($n = 6$) from two experiments. Differences were statistically significant by one-way ANOVA. Post hoc analysis with Newman–Keuls Multiple Comparison Test demonstrated that inhibition of Bax ameliorates DHT-mediated apoptosis at day 5. ***$P < 0.001$ (vs. control); #$P < 0.001$ (vs. DHT control). (C) Western blot analysis for altered Bcl-2 and Bax expression. Proteins from total cellular extracts isolated 48 h after mock transfection or cultures transfected with Bcl-2 expression plasmid (left panel) or control or Bax siRNAs (right panel) were analyzed by Western blot probed with Bcl-2 or Bax antibody. Equal protein loading was confirmed by analysis with α-tubulin antibody. Quantification of immunoband intensities was determined by densitometric scanning. The band intensity values of Bcl-2 or Bax were normalized with respect to the band intensity values of α-tubulin. −, mock-transfection; con, control siRNA oligonucleotides; +, bax siRNA oligonucleotides.

Previously, characterization of the effects of androgen treatment on osteoblast apoptosis in vivo has been only performed in recently orchidectomized animals in a setting of high turnover and activated resorption [27], rather than associated with bone growth in an anabolic circumstance. We therefore characterized apoptosis in male AR-transgenic animals with TUNEL staining in calvarial cross sections. Post-pubertal (Fig. 7C, left panels) and adult (Fig. 7C, right panels) male AR-transgenic mice showed increased TUNEL staining when compared to wild-type controls, both in areas of new bone growth (proliferating osteoblasts). TUNEL staining was also demonstrated in matrix-embedded osteocytes in calvarial sections where new bone growth was not evident (Fig. 7D). Negative controls verified a lack of staining (see inset). Higher power magnifications of the apoptotic cells (see insets) identify the apoptotic cells as

osteoblastic and not multi-nucleated osteoclasts, based on location and morphology.

Discussion

In this study, we explored the specific role of androgen, contrasted with estrogen, in the regulation of osteoblast apoptosis using in vivo and in vitro models of enhanced androgen responsiveness. We have previously demonstrated that chronic androgen treatment reduces osteoblast viability in both normal primary cultures and in clonal colAR-MC3T3 cultures, an effect mediated via inhibition of the ERK cascade [57]. Consistent with those findings, we report here that continuous treatment with the non-aromatizable androgen DHT stimulated, while similar treatment with E_2 reduced, osteoblast apoptosis. Androgen treatment enhanced osteoblast apoptosis in
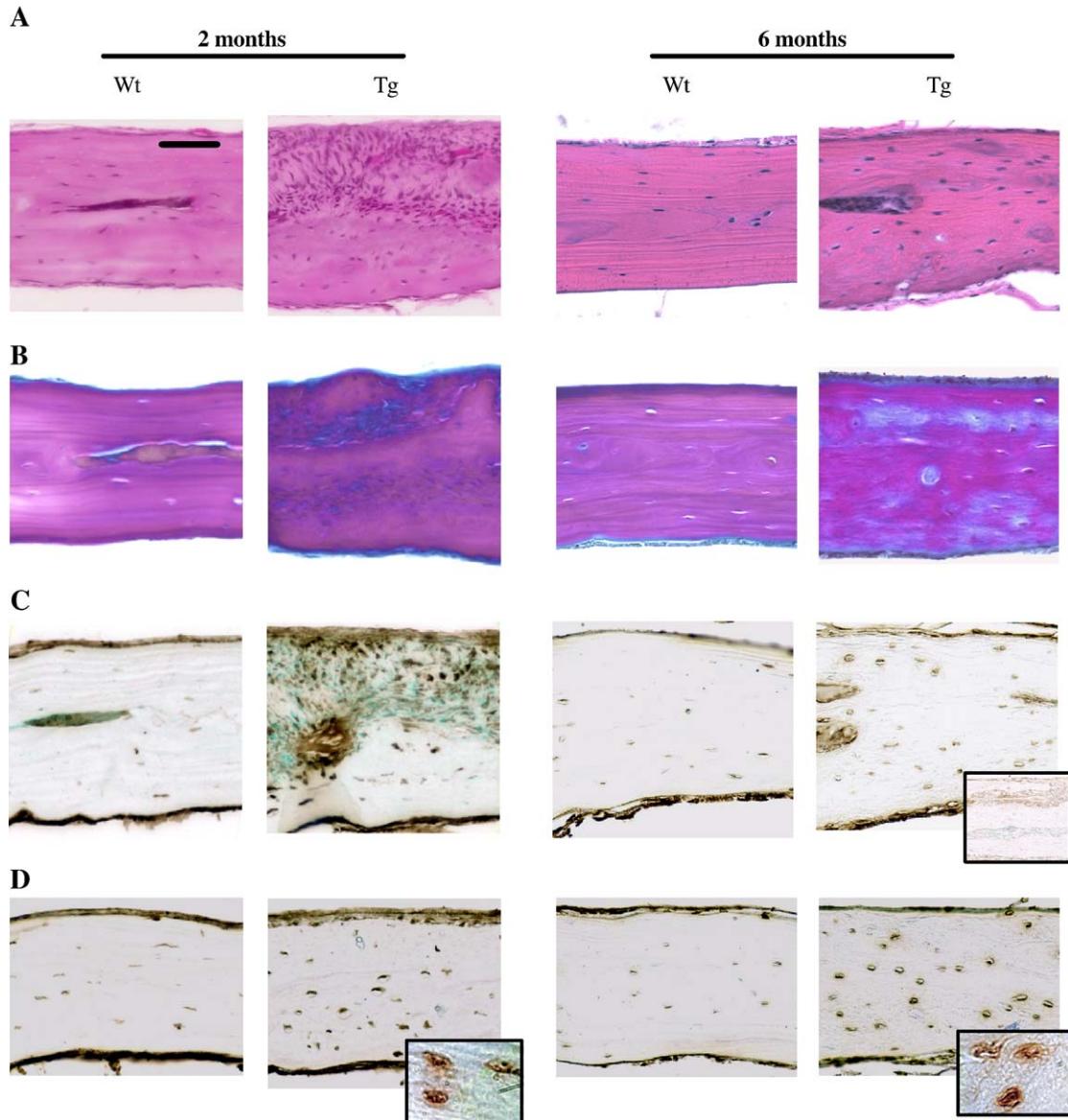
Fig. 7. Characterization of osteoblast apoptosis in vivo using AR-transgenic mice. Calvaria from either 2 month old (left panels) or 6 month old (right panels) male AR-transgenic or littermate control mice were fixed, decalcified, embedded, and calvarial cross sections were evaluated. (A) Calvarial histology. H&E stained cross sections show calvarial thickening in male AR-transgenic mice. Scale bar indicates 50 μm. (B) New bone growth. New bone growth associated with new collagen synthesis was identified in calvarial cross sections using van Geison staining. New collagen synthesis is shown in post-pubertal 2 month old mice and in adult 6 month old mice. (C) Analysis of apoptosis with TUNEL staining with new bone formation. In situ end-labeling (TUNEL) staining was performed for analysis of apoptosis and counterstained with methyl green. Sections were derived from areas demonstrating new bone formation as described in panel C. Negative control sections for TUNEL analysis were incubated without TdT (inset). Quantitative analysis indicated ∼10% apoptotic cells in wild-type and ∼40% in AR-transgenic mice. (D) Analysis of apoptosis with TUNEL staining in osteocytic cells. TUNEL staining was performed in calvarial outer regions that do not demonstrate thickening. Enhanced apoptosis was observed in male AR-transgenic mice both in areas with new bone formation and in matrix-embedded osteocytes. Higher power magnification shows osteoblasts morphology (inset). wt, wild-type mice (left panels); tg, AR-transgenic mice (right panels). Microscope magnification at 40× with higher power inset image at 100×.

both proliferating and mature osteocyte-like cells in vitro, and the effects were abrogated in the presence of an androgen receptor antagonist. Levels of the antiapoptotic regulator Bcl-2 were reduced post-transcriptionally by androgen treatment, associated with enhanced ubiquitination. Reduced serine phosphorylation of Bcl-2 was also observed, consistent with inhibition of ERK1/2 activity. Notably, the increased Bax/Bcl-2 ratio was necessary for androgen-enhanced apoptosis since overexpression of *bcl-2* or siRNA knockdown of *bax* abrogated

the effects of DHT. In vivo analysis of calvarial sections from skeletally targeted male AR-transgenic mice at two time points also demonstrated enhanced TUNEL staining in osteoblasts when compared with wild-type controls, even in areas of new bone growth. Collectively, these data indicate that enhanced osteoblast apoptosis after androgen exposure is mediated by an increase in the Bax/Bcl-2 ratio via a reduction in Bcl-2 phosphorylation and MAP kinase activation, and is observed even in anabolic settings.

Notably, an association of osteoblast apoptosis with osteogenesis has been observed previously. During bone formation, osteoblasts undergo an orderly developmental progression ultimately ending in apoptosis [19]. It has been estimated that as many as 65% of osteoblasts undergo apoptosis after completing synthesis of bone matrix [25], suggesting that apoptosis is a fundamental component of the osteoblastic differentiation program [31]. TUNEL-positive osteoblasts and even preosteoblasts have been demonstrated at or close to the osteogenic fronts in areas of intense osteogenic activity during calvarial growth [40]. In addition, caspase-3-deficient mice show delayed ossification and decreased bone mineral density, suggesting that caspase activity plays a critical role in osteogenic differentiation in vivo [34]. Finally, bone-targeted Bcl-2 overexpression leads to reduced bone formation in a sex-specific fashion [38]. Thus, apoptosis can be associated with osteogenesis and the homeostatic maintenance of bone. This point is worth noting, since androgen-mediated apoptosis might be inappropriately viewed as inconsistent with anabolic actions.

In some tissues, androgen treatment has been reported to enhance apoptosis [54,64] and decrease Bcl-2 levels [6,20,30]. In this report, we show that treatment with DHT in osteoblasts also results in reduced Bcl-2 protein levels and enhanced apoptosis. Bcl-2 protein can be stabilized upon ERK1/2 activation in some settings [8,14,41]. While it has been reported that Bcl-2 phosphorylation can also facilitate apoptosis [60], it may also be that Bcl-2 phosphorylation can enhance survival but is not sufficient to prevent cell death with continuous toxin exposure [8]. Consistent with our studies, dephosphorylation of Bcl-2 results in enhanced apoptosis, mediated by ubiquitin-induced degradation of Bcl-2 [5,9]. These reports suggest a link between MAP kinase activity and the proteasome pathway to regulate Bcl-2 levels. Although three consensus MAP kinase sites have been identified in Bcl-2 [5], Bcl-2 is the target of multiple kinases including c-Jun N-terminal kinase 1, protein kinase A, and PKCα [8,23]. Furthermore, Bcl-2 phosphorylation is a dynamic process that is negatively regulated by protein phosphatase 2A [21]. In addition, the antiapoptotic effect of growth factor signaling may be mediated by additional proteins through PI3 kinase-mediated pathways [53]. Thus, it is likely that the regulation of the antiapoptotic function of Bcl-2 by phosphorylation is cell-type-specific and may be dependent on additional factors that are as yet undefined. Here, we demonstrate that, in osteoblasts, androgen treatment decreases Bcl-2 protein levels post-transcriptionally with reduced serine phosphorylation and increased ubiquitination. Thus, one mechanism through which androgen treatment enhances osteoblast apoptosis may be via inactivation of the antiapoptotic function of Bcl-2 by reducing Bcl-2 protein level through ubiquitin-mediated proteolytic degradation, as expected with suppressed ERK1/2 activity [57].

The antiapoptotic effect of estrogen and the proapoptotic effect of androgen treatment were observed both in proliferating osteoblasts and in mature osteocytic cultures. These results are supported by a recent publication of opposing effects of estrogen and androgen on apoptosis, here in T47D cells [22]. Further, our in vitro analyses are consistent with multiple reports indicating that estrogen can reduce osteoblast apoptosis in some [12,27,65], but not all circumstances [11]. Neither Bcl-2 nor Bax expression was significantly influenced by E_2 treatment alone, contrary to the effects of androgen treatment. In the presence of apoptotic stimuli such as glucocorticoid treatment, E_2 is able to prevent the increased Bax/Bcl-2 ratio [12,13,38]. To our knowledge, this is the first report demonstrating divergent and distinct regulation by androgen vs. estrogen with regard to osteoblast apoptosis. These results again indicate that non-specific non-genomic signaling through either ERs or AR, reportedly activated with the same effectiveness regardless of whether the actual ligand is an androgen or an estrogen [27], is not a significant modulator of osteoblast apoptosis induced by androgen treatment. Analysis with AR overexpression in Fig. 1C is also consistent with mediation of androgen action strictly by AR transactivation, and not through non-specific interactions with ERs nor through generalized squelching of steroid receptor-mediated signaling.

A contrasting response to estrogen and androgen administration has also been described in some bone compartments in vivo. This is particularly true in cortical bone, where at the periosteum estrogen suppresses but androgen stimulates new bone formation [49], while at the endosteum estrogen stimulates but androgen strongly suppresses formation [59]. These envelope-specific responses likely play an important role in determining sexual dimorphism of the skeleton, i.e., that male bones are wider but not thicker than females [43]. In trabecular bone, ER and AR signaling may have functional redundancy, although not with exactly the same mechanism [36]. Combined, these results suggest that administration of both estrogen and androgen may be beneficial, and is consistent with clinical studies demonstrating that combination therapy with estrogen and androgen is more beneficial than either steroid alone in post-menopausal women [7,33], recently confirmed in an animal model [46]. Ongoing analysis examining the effects of both steroids in combination will be important for a better understanding of the impact of combination therapy on bone homeostasis.

Few studies have characterized the specific effect of androgen in vivo on osteoblast apoptosis. In one report, increased osteoblast apoptosis was seen after orchidectomy in adult mice (where both estrogen and androgen levels are reduced). Six weeks of DHT treatment in these orchidectomized mice was associated with reduced osteoblast apoptosis in vertebrae [27], a site of predominantly trabecular bone. However, in this setting soon after orchidectomy, bone resorption is dramatically increased and thus one cannot adequately evaluate a connection between anabolic effects of androgen treatment associated with new bone growth and osteoblast apoptosis. Results reported here are also in contrast to the in vitro response noted after short-term DHT treatment that demonstrated inhibition of apoptosis [27]. While the reasons for the discrepancy are not clear, inhibition of apoptosis reported in this setting may have been a consequence of stimulation of ERK activity associated with rapid non-genomic steroid actions, since longer androgen treatment results in reductions in MAP kinase activation [57]. Interestingly, it is possible that some

aspects of the anabolic response to androgen we and others have described could be the result of an increase in osteoblast cell proliferation via a transient non-genomic androgen-mediated increased MAP kinase, PKC or PI3 kinase, or other kinase cascades and/or calcium mobilization (e.g., see [24]), through potentiation of growth factor and/or cytokine signaling, or through an as yet unexplained mechanism. Androgen treatment has been reported to increase osteoblast proliferation in vitro when treatment times are short [57] or transient (e.g., 15 min, see [24]), and to increase collagen expression [3]. The anabolic response is likely to be complicated, however, since there is no clear mechanism to block androgen signaling after transient induction in vivo. In addition, osteogenesis in vivo is generally not associated with estrogen treatment that does increase MAP kinase activity [10].

In conclusion, we have demonstrated that chronic DHT treatment in both proliferating and in mature osteocytic cultures resulted in enhanced osteoblast apoptosis. This result contrasts with the inhibitory effects on apoptosis observed with E_2 treatment. An androgen-mediated increase in the Bax/Bcl-2 ratio was observed, predominantly through inhibition of Bcl-2. Increased Bax/Bcl-2 was necessary and sufficient for androgen-enhanced apoptosis since overexpression of *bcl-2* or RNAi knockdown of *bax* abrogated the effects of DHT, and was dependent on functional AR. These data suggest that enhanced apoptosis is mediated by an increase in the Bax/Bcl-2 ratio, at least in part as a consequence of reductions in Bcl-2 phosphorylation and protein stability consistent with inhibition of MAP kinase pathway activation. Analysis of calvaria in AR-transgenic male mice demonstrated enhanced TUNEL staining in vivo even in areas of new bone growth. In bone, apoptosis in osteoblasts has been reported in vitro and during development in vivo [31]. As has been observed in other remodeling tissues and/or associated with development and tissue homeostasis [29], mounting evidence has identified an association between new bone growth and apoptosis [37]. Apoptotic cell death could thus be important in making room for new bone formation and matrix deposition, which may have clinical significance by influencing bone homeostasis and bone mineral density [34]. Collectively, the findings reported here help to resolve a controversy surrounding the role of sex steroids in bone homeostasis by demonstrating that androgen directly stimulates while estrogen inhibits osteoblast apoptosis, and suggests that modulation of osteoblast apoptosis may be an important consequence of both androgen and estrogen signaling but with distinct outcomes. These data demonstrate that androgen signaling through the AR in bone directly influences osteoblast function in growing tissue in vivo and offer valuable insight into the role of androgen signaling in bone homeostasis. Further studies on androgen action in the developing skeleton, during repair and in the adult are warranted.

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The Effect of Oxandrolone Treatment on Human Osteoblastic Cells

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Objective: Oxandrolone, administered to severely burned children over the first year
postburn, produces increased lean body mass by 6 months; however, an increase in to-
tal body bone mineral requires 12 months. Consequently, this bone mineral response
may be due to increased muscle mass. Alternatively, oxandrolone may act directly on
bone. The current study seeks to determine whether oxandrolone can transactivate the
androgen receptor in osteoblasts. **Methods:** Collagen, alkaline phosphatase, osteocal-
cin, osteoprotegerin, and androgen receptor abundance were determined by qRT-PCR,
confocal laser scanning microscopy, or immunoquantitative assay. To determine the ef-
fect of oxandrolone on gene expression in differentiated cells, osteocytic cultures were
grown to confluence in differentiation medium and then treated 24 hours or 5 days
with 15 μg/mL oxandrolone. **Results:** Increased nuclear fluorescence of the androgen
receptor and increased cellular type I collagen were observed with oxandrolone at 15
and 30 μg/mL but not at lower doses. Alkaline phosphatase (7%–20%) and osteocalcin
(13%–18%) increases were modest but significant. Short-term treatment produced no
significant effects, but at 5 days androgen receptor levels were increased while collagen
levels were significantly decreased, with little effect on alkaline phosphatase, osteocal-
cin, or osteoprotegerin. **Conclusions:** These data suggest oxandrolone can stimulate
production of osteoblast differentiation markers in proliferating osteoblastic cells, most
likely through the androgen receptor; however, with longer treatment in mature cells,
oxandrolone decreases collagen expression. Thus it is possible that oxandrolone given to
burned children acts directly on immature osteoblasts to stimulate collagen production,
but also may have positive effects to increase bone mineral through other mechanisms.

Long-term use of the orally administered anabolic agent oxandrolone has been shown
to increase both lean body mass and bone mineral content in severely burned children when

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given over the first year postburn.[1] Oxandrolone and recombinant human growth hormone[2] were shown to be effective in recovering bone that would ordinarily be lost following the burn injury, resulting in markedly reduced bone formation[3] hypocellularity at the mineralization front of bone[3,4] and decreased marrow stromal cell differentiation into osteoblasts.[4]

Increased endogenous glucocorticoid production is likely responsible for the acute bone loss observed in severely burned patients.[3,4] Notably, with both oxandrolone and recombinant human growth hormone, an increase in lean body mass precedes an increase in bone mineral content by 3 to 6 months.[1,2] Thus it is not clear whether these anabolic agents increase bone mineral content secondary to increased muscle mass and hence increased skeletal loading, or whether they act directly on osteoblastic cells. Oxandrolone is an anabolic steroid with the ability to transactivate the androgen receptor (AR), which may be one mechanism underlying the anabolic response to therapy. The aim of our study is to determine whether oxandrolone can increase osteoblastic production of type I collagen and whether this action is mediated by AR signaling.

MATERIALS AND METHODS

Human osteoblast cell cultures

Freshly discarded human cancellous bones were obtained from healthy young patients undergoing osteotomy. The bone fragments were washed with serum-free α–minimum essential medium (α-MEM, Flow Laboratories, McLean, VA). Then, the fragments were digested in the medium with 1 mg/mL collagenase for 2 hours at 37°C. The enzymatic reaction was stopped by adding an equal volume of α-MEM with 10% fetal bovine serum (FBS). The supernatant containing the released cells was recovered. Washing and recovering were repeated 3 times. The cells were transferred to a centrifuge tube and centrifuge 10 minutes at $100 \times g$ to harvest the cells. The cell pellet was resuspended in 5 mL of fresh medium. The single cell suspension was cultured in α-MEM containing antibiotics [penicillin (100 U/mL), streptomycin sulphate (100 μg/mL)] and 10% FBS in a humidified incubator at 37°C under an atmosphere of 5% CO_2 and 95% air. After confluence, adherent cells were collected by trypsinizing with 0.025% trypsin-EDTA and resuspended in α-MEM. Only passages 4 to 8 were used in this study. Medium was changed every other day.[4]

Osteocytic cell culture

Osteoblasts in vitro progress through several developmental stages that correlate with osteoblast development in vivo: from committed preosteoblasts to mature, differentiated osteoblasts, and finally to osteocyte-like cells embedded in mineralized extracellular matrix.[5–7] To characterize the effects of oxandrolone in osteocytic cells, human osteoblast cells were cultured in α-MEM with 5% fetal bovine serum containing antibiotics for 10 days until confluence, then switched to BGJ_b medium containing 50 μg/mL ascorbic acid and 3 mM β-glycerol phosphate.[7] Cultures were maintained in differentiation medium for 7 days for mature osteoblast/osteocyte development,[7] then treated with oxandrolone for time course and dose-response analysis. These highly confluent osteocytic cultures were treated in 5% charcoal-stripped serum with either 15 μg/mL of oxandrolone for 24 hours or 5 days, or

with increasing concentrations of oxandrolone (0, 1, 5, 10, and 15 μg/mL) for 5 days. Total RNA was isolated for gene expression analysis as described below.

Immunohistochemistry for collagen type I and AR

Immunohistochemical staining was carried out for both the AR[8,9] and type I collagen. Human osteoblastic cells were trypsinized, seeded on slide chambers (Lab-Tek, Nalge Nunc International, Rochester, NY) at a concentration of 1×10^5 cells/mL and cultured for 3 days until they achieved 60% to 70% confluence. Oxandrolone was used to stimulate the cells at the concentration of 1, 5, 10, 15, and 30 μg/mL for 24 hours. Stock solution containing 2.5 mg diluted in 2 mL of DMSO was prepared and subsequently diluted in culture media, while control nonstimulated cells received the same solution without oxandrolone. Thereafter, the medium was discarded, the cells were washed with phosphate buffered saline (PBS), and fixed in $-20°$C methanol/acetone (50:50) for 20 minutes and permeabilized with 0.5% Triton X-100 (Sigma, St Louis, MO). The polyclonal antibody used for collagen I was applied overnight at the dilution of 1:200 (Research Diagnostics Inc, Flanders, NJ). The polyclonal antibody for AR (Neomarkers, Fremont, CA) was used at the dilution of 1:30. The next day, FITC-conjugated goat anti-rabbit IgG was used as secondary antibody (Neomarkers) and cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Vector Mounting Medium, Burlingame, CA). Images were captured using the laser confocal scanning microscope (Zeiss LSM 510, Jena, Germany) with a $63\times$ objective. Optical sections were obtained from the cells by capturing single images of central cell focal planes. Three microscopic fields were captured for the control group of each cell line and the same was done for cells treated with oxandrolone. To proceed to an analysis of AR immunohistochemistry, the intensity of fluorescence was measured in the nuclei (AR) in 2 cells per 3 fields, using Image Tool software (University of Texas Health Science Center, San Antonio).

Assay of alkaline phosphatase

Human osteoblastic cells were cultured in α-MEM containing antibiotics and 10% FBS. The cells were challenged with increasing concnetrations of oxandrolone (0, 1, 5 10, 15 μg/mL). After 24 hours of treatment, the levels of alkaline phosphatase activity were determined using a commercial kit (Pierce Biotechnology, Inc, Rockford, IL). The cells were washed with cold PBS and subjected to 3 freeze-thaw cycles. These samples were assayed for enzymatic activity with p-nitrophenyl phosphate as a substrate. Sample absorbance was measured at 400 nm with microplate reader. Results were expressed in ALP (OD)/protein (OD).

Immunoquantitative assay for collagen type I and osteocalcin

To perform the immunoquantitative assay for type I collagen[4] and osteocalcin, human osteoblastic cells were subcultured on 96-well plates until they achieved 70% confluence. Oxandrolone was used to stimulate the cells at the concentration of 0, 1, 10, 15, and 30 μg/mL for 24 hours. Cells were harvested and washed with cold PBS, fixed in methanol for 10 minutes at $-20°$C and washed with PBS. They were then preincubated with primary antibody for type I collagen and osteocalcin (Santa Cruz Biotechnology Inc, CA), respectively, overnight at 4°C, followed by secondary biotinylated IgG for 30 minutes at room temperature. After washing with PBS, cells were exposed to p-nitrophenyl phosphate containing levamisole to inhibit the generation of p-nitrophenol by endogenous alkaline phosphatase

for 8 minutes. Sample absorbance was measured at 400 nm with an ELISA reader. The values (OD) were normalized with protein concentration (OD).

Real-time quantitative reverse transcription–polymerase chain reaction (qRT-PCR) on cultured human osteocytic cells

Total RNA was isolated from osteocytic cultures and gene expression characterized by qRT-PCR analysis using human primers from Qiagen (Valencia, CA) specific for AR and the osteoblast marker proteins alkaline phosphatase, type I collagen, osteocalcin, and osteoprotegerin. The qRT-PCR analysis was performed with the iCycler IQ Real Time PCR detection system (Bio-Rad Laboratories, Inc, Hercules, CA) using a one-step QuantiTect SYBR Green RT-PCR kit (Qiagen) on DNase-treated total RNA. Relative expression of the RT-PCR product was determined using the comparative $\Delta\Delta C_t$ method after normalizing expression with fluorescence to the specific RNA-binding dye RiboGreen (Molecular Probes, Eugene, OR) as previously described.[10] Real-time qRT-PCR efficiency was determined for each primer set using a fivefold dilution series of total RNA and did not differ significantly from 100%. Individual reaction kinetics were also analyzed to ensure each real-time RT-PCR did not differ significantly from 100% efficiency. Following PCR, specificity of the PCR reaction was confirmed with inverse derivative melt curve analysis. Data is presented as mean $\pm$ SEM.

Statistical analyses

Data are reported as mean $\pm$ SEM. The statistical significance of intergroup differences was tested using the Student t test when the variances were equal. P less than .05 was considered significant.

RESULTS

Oxandrolone treatment in proliferating human osteoblastic cells

Proliferating cultures of normal human osteoblastic cells were treated for 24 hours with 30 μg/mL oxandrolone or vehicle to determine the acute response to oxandrolone treatment. Although the affinity for oxandrolone for AR is approximately 100-fold lower than testosterone, oxandrolone treatment still results in AR transactivation.[11] To characterize the ability of oxandrolone to stimulate translocation of the AR to the nucleus, confocal laser scanning microscopy was employed. When control cultures were stained for AR, diffuse cytoplasm staining but only weak nuclear staining were observed on osteoblasts nonstimulated with oxandrolone (Fig 1a). After stimulation with oxandrolone, increased fluorescence of AR in the nuclei was seen (Fig 1b), suggesting transactivation by oxandrolone at these concentrations.

We next assessed the effect of oxandrolone treatment on type I collagen, the major constituent of the bone matrix. Immunohistochemistry for collagen type I on cultured osteoblast cells demonstrated an increase in collagen that was dose dependent. When compared to control group (Fig 1c), a significant increased intensity of fluorescence was seen for cells stimulated with oxandrolone. This was observed when the concentration of oxandrolone was 15 μg/mL (Fig 1f) and 30 μg/mL (Fig 1d).
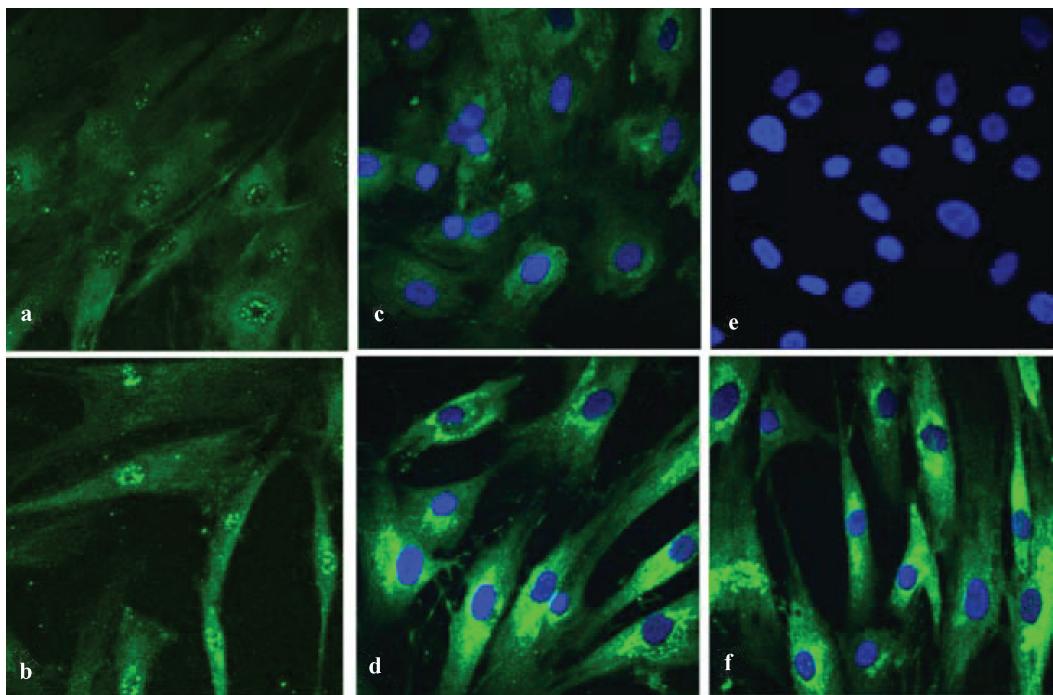
Figure 1. Confocal scanning laser microscopic analysis of human osteoblastic cells after oxandrolone treatment. (a) Confocal scanning laser microscopy depicting AR nuclear fluorescence of human osteoblastic cells not stimulated with oxandrolone, (b) confocal scanning laser microscopy depicting the increased AR nuclear fluorescence of human osteoblastic cells stimulated with oxandrolone 30 μg/mL, (c) confocal scanning laser microscopy depicting cytoplasmic fluorescence of type I collagen in human osteoblastic cells not stimulated with oxandrolone, (d) confocal scanning laser microscopy depicting increased cytoplasmic fluorescence of type I collagen in human osteoblastic cells stimulated with oxandrolone 30 μg/mL, (e) internal negative control for primary antibodies of AR and type I collagen, (f) confocal scanning laser microscopy depicting increased cytoplasmic fluorescence of type I collagen in human osteoblastic cells stimulated with oxandrolone 15 μg/mL. Compare to Figures 1c and 1d.

To determine the effect of oxandrolone on additional markers of osteoblast activity, osteoblastic cultures were treated with or without oxandrolone. Alkaline phosphatase activity was determined in cells treated with oxandrolone for 24 hours (Fig 2), and there was an increase (7%–20%) in activity in a dose-dependent manner. The differences were statistically significant ($P < .05$) but the changes were minor compared to the control group. We also performed immunoquantitative assays for type I collagen and osteocalcin on osteoblastic cells. In cultures treated with oxandrolone (10 or 15 μg/mL), collagen was increased 21% to 35% (Fig 3; $P < .05$). There were no differences in the groups treated with low concentrations of oxandrolone (1 or 5 μg/mL) compared to control group. Thus, after multiple repetitions of the experiment, the data confirmed the results observed using immunofluorescence.

We next assessed the levels of osteocalcin, an important biochemical indicator of bone turnover.[12] Cells treated with oxandrolone of 10, 15, and 30 μg/mL produced a greater level of osteocalcin (11%–18%) compared to the control group. The differences were statistically
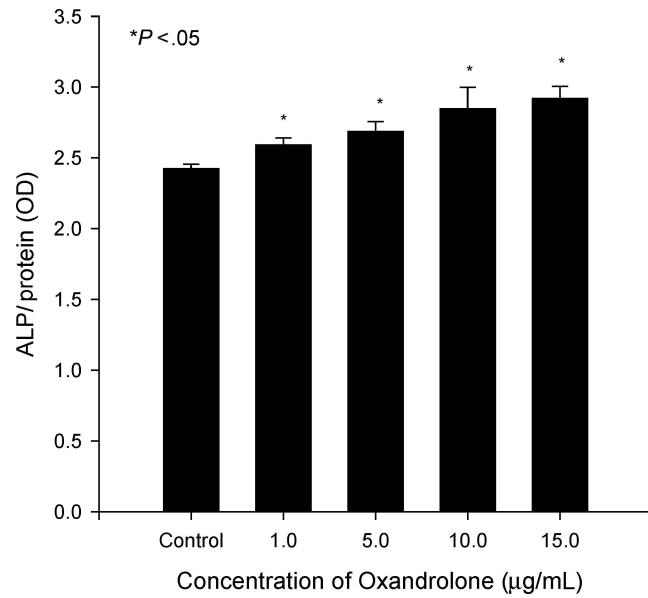
Figure 2. Stimulation of alkaline phosphatase activity by oxandrolone in proliferating human osteoblastic cultures. Proliferating cultures were treated acutely for 24 hours with 0, 1, 5, 10, and 15 μg/mL oxandrolone. Data are expressed as mean $\pm$ SEM of 6 determinations.
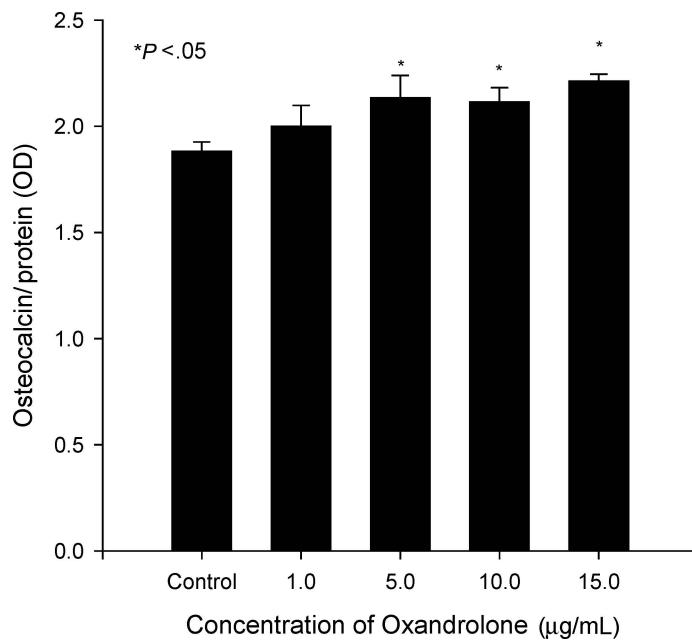
Figure 3. Increased osteocalcin levels after oxandrolone treatment. Human osteoblastic cultures were exposed to oxandrolone (0, 1, 5, 10, and 15 μg/mL) for 24 hours. Data are expressed as mean $\pm$ SEM of 6 determinations.
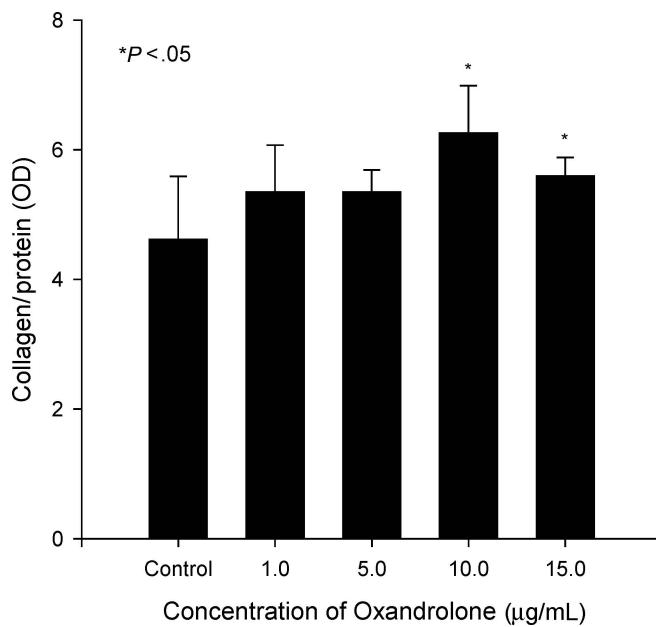
Figure 4. Enhanced type I collagen secretion after oxandrolone exposure. Proliferating cultures were treated with 0, 1, 5, 10, and 15 μg/mL oxandrolone for 24 hours. Data are expressed as mean $\pm$ SEM of 6 determinations.

significant (Fig 4; $P < 0.05$) but again the increase was small. Thus, the production of osteocalcin mirrored the pattern of alkaline phosphatase activity.

AR concentrations increase as osteoblasts differentiate, reaching their highest levels in osteocytic cultures.[13] Furthermore, the most abundant cell in bone is the osteocytic cell. We therefore determined the effect of oxandrolone treatment on gene expression in mature osteocytic cultures. Normal human osteoblasts were cultured for 10 days, then switched to differentiation medium containing ascorbic acid and β-glycerol phosphate. After 7 days, osteocytic cells were treated with 15 μg/mL oxandrolone. Total RNA was isolated after 24 hours or 5 days treatment, and gene expression characterized by qRT-PCR analysis using human primers specific for AR, type I collagen, alkaline phosphatase, osteocalcin, and osteoprotegerin (Fig 5). Although there was little effect of oxandrolone after 24 hours of treatment in osteocytic cells on type I collagen, alkaline phosphatase, osteocalcin, or osteoprotegerin mRNA abundance, a nonsignificant increase in AR mRNA was noted. This is consistent with other reports documenting an increase in AR after androgen treatment in osteoblasts.[14] After stimulation with oxandrolone for 5 days, a significant decrease in expression of type I collagen was seen ($P < 0.01$). Thus, longer treatments with oxandrolone in osteocytic cultures reduced collagen expression. These data are consistent with dose-response studies performed after 5 days of treatment with increasing concentrations of oxandrolone (Fig 6), where AR mRNA is modestly increased but collagen levels are significantly decreased in a dose-dependent fashion with oxandrolone treatment.
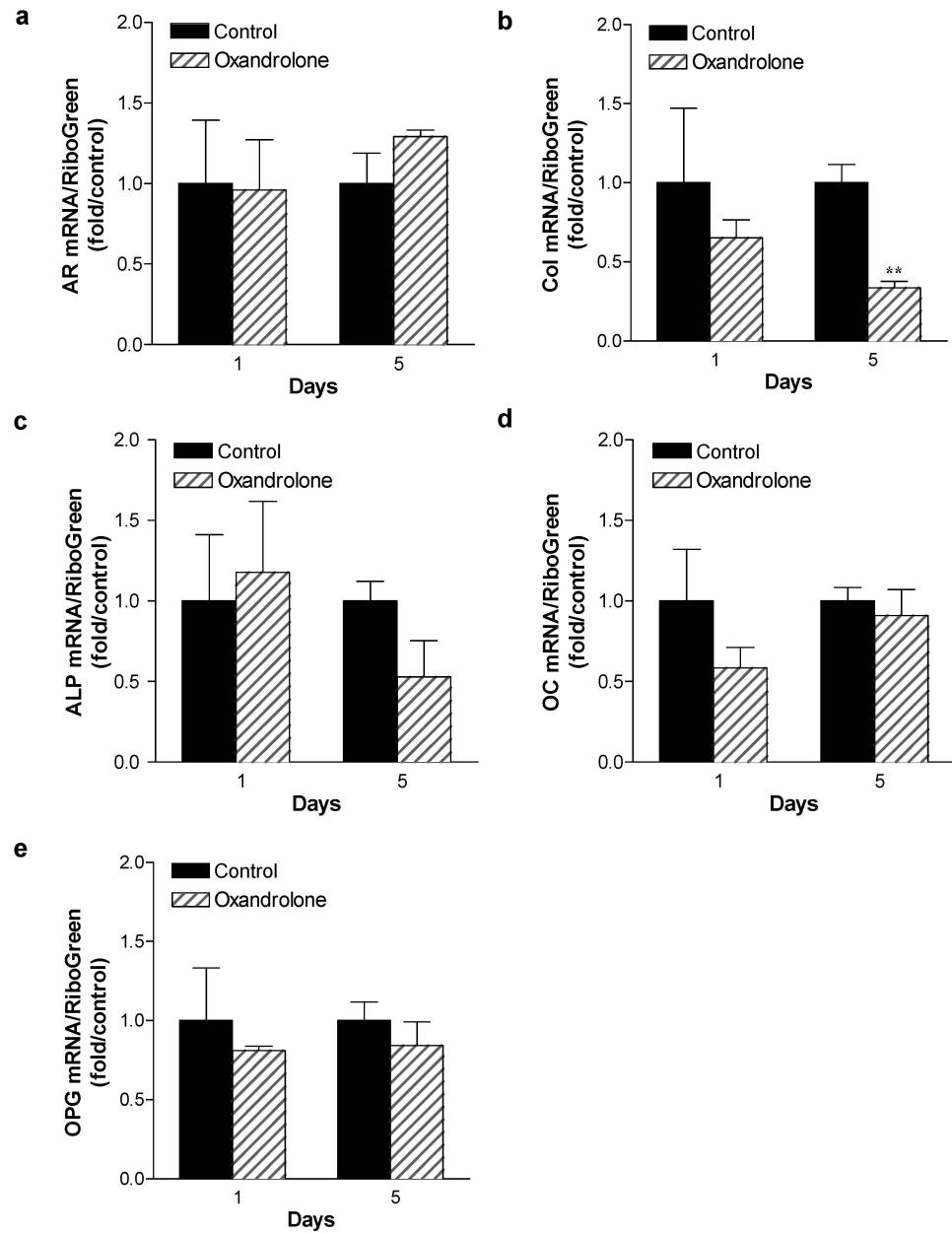
Figure 5. Consequence of oxandrolone treatment on gene expression in normal human osteocytes: time course analysis. Normal human osteoblastic cells were cultured for 10 days to confluence, then switched to differentiation medium containing ascorbic acid and β-glycerol phosphate. After 7 days, osteocytic cells were treated with 15 μg/mL oxandrolone. Total RNA was isolated after 24 hours or 5 days treatment, and gene expression characterized by qRT-PCR analysis using human primers specific for AR, type I collagen (col), alkaline phosphatase (ALP), and osteocalcin (OC) and osteoprotegerin (OPG). $n = 3$ to 4. **$P < .01$.
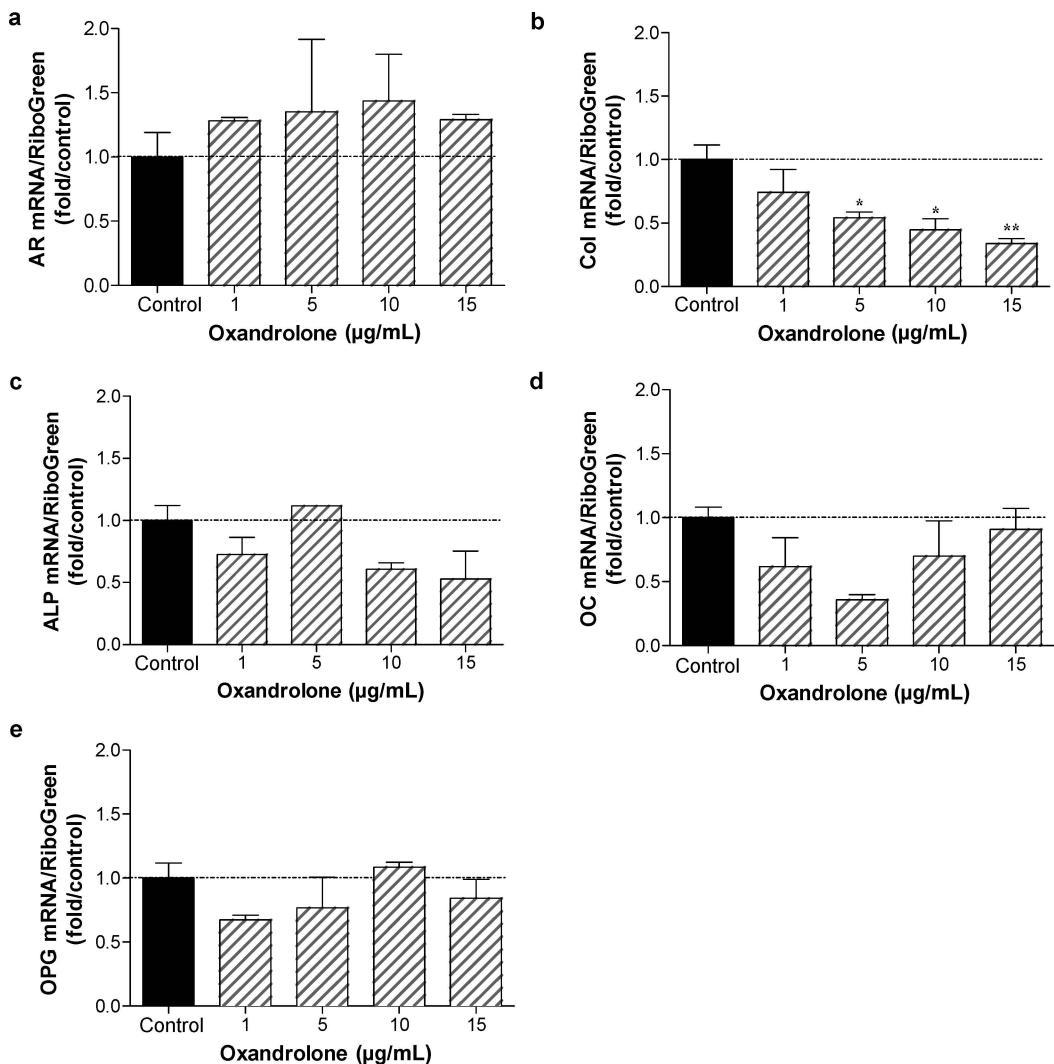
Figure 6. Consequence of oxandrolone treatment on gene expression in normal human osteocytes: dose-response analysis. Normal human osteoblastic cells were cultured as described in Figure 5. Osteocytic cultures were treated for 5 days with 0, 1, 5, 10, and 15 μg/mL oxandrolone. Total RNA was isolated and gene expression characterized by qRT-PCR analysis using human primers specific for AR, type I collagen (col), alkaline phosphatase (ALP), osteocalcin (OC) and osteoprotegerin (OPG). $n = 2$ to 4. *$P < .05$; **$P < .01$.

DISCUSSION

In this study we have shown that stimulation of cultured human osteoblasts by oxandrolone results in immunofluorescent detection of AR in the nucleus and increased osteomarkers in these osteoblasts. These data suggest that oxandrolone directly targets human osteoblasts by means of the AR, resulting in increased expression of osteoblast differentiation markers after short-term treatment. Therefore, oxandrolone may act directly on the osteoblast in addition to effects that result in increasing skeletal loading.

Immunohistochemistry and confocal laser scanning microscopy (CLSM) were used to evaluate the expression of AR and type I collagen in osteoblasts, with several advantages over regular fluorescence microscopy.[15,16] While in conventional wide-field fluorescence microscopy the emitted light coming from regions above and below the focal plane is collected by the objective lens and contributes to an out-of-focus blur in the final image, in the CLSM a diaphragm rejects the out-of-focus information.[15,16] Therefore, tissue thickness does not interfere with the resulting fluorescence. The increased expression of type I collagen by osteoblasts was also determined by immunoassay confirming the results observed with immunohistochemistry.

The AR is a steroid receptor that generally mediates biologic responses to androgens. The increased expression of AR in the present study is consistent with that documented in the literature.[14] In bone tissue the AR is expressed in a variety of cell types, but its specific role in the maintenance of skeletal homeostasis remains controversial.[17] In an experimental study, androgen deficiency was shown to result in a substantial loss of cancellous bone in the axial and appendicular skeleton of aged male rats and that this osteopenia is associated with a sustained increase in bone turnover.[18] Consistent with this, the bone phenotype that develops in a global AR null (ARKO) male mouse model is a high-turnover osteopenia, with reduced trabecular bone volume, and a significant stimulatory effect on osteoclast function.[19,20]

Alkaline phosphatase is well recognized as a marker that reflects osteoblastic activity.[21] Kasperk et al reported that androgens increase the alkaline phosphatase activity in osteoblast culture.[22–24] However, there are also reports of androgens either inhibiting[25] or having no effect on alkaline phosphatase activity,[26,27] which may reflect both the complexity of osteoblastic differentiation and the various model systems employed. In a clinical study, Murphy et al[1] have shown that oxandrolone administration increases levels of serum alkaline phosphatase in treated patients by 6 months versus controls. In the present study, the cells treated with oxandrolone produced a greater level of alkaline phosphatase. The elevated activity of this cellular enzyme suggests an early increase in the osteoblast differentiation process.

Osteocalcin appears to be a bone-specific gla-containing protein, accounting for 10% to 20% of the noncollagenous protein in bone. While the in vivo function of osteocalcin remains unclear, its affinity for bone mineral constituents implies a role in bone formation. Hence it has been shown that osteocalcin is a biochemical indicator of bone turnover.[12] In our study, proliferating cells treated for 24 hours with oxandrolone produced small but statistically significant increases in osteocalcin production compared to control group.

To test the effect of oxandrolone on osteocyte-like cells differentiated from osteoblasts at the molecular level, the human osteoblastic cells were cultured in a differentiation medium and then exposed to oxandrolone. The results showed that short-term treatment produced little effect on type I collagen, alkaline phosphatase, osteocalcin, osteoprotegerin, or AR mRNA. Long-term treatment decreased type I collagen expression, consistent with decreased collagen expression observed by Wiren et al[16] in AR-transgenic mice with targeted AR overexpression in the osteoblast lineage.[16] The results described here are consistent with observations reported in vivo with male AR-transgenic mice, where calvarial thickening is observed and cortical formation is altered with surface periosteal expansion but inhibition of inner endosteal deposition, consistent with the known effects of androgen to stimulate periosteal apposition. The dramatic inhibition at the endosteal envelope may be

responsible for a modest decrease in cortical bone area and reductions in biomechanical properties observed.[16] Thus, taking all data together, our results suggest that osteoblast cells are targeted by androgens to transactivate AR in bone.

Murphy et al[1] have recently shown that oxandrolone administration increases lean body mass 3 to 6 months before an increase in bone mineral content is observed. On the other hand, we have found an increase in collagen production when high doses of oxandrolone were used to stimulate osteoblasts. The applicability of our findings in this study to the in vivo effects of oxandrolone in burned children[1] is not clear. While an in vitro environment may not adequately reproduce the in vivo situation, many potential variables are eliminated during in vitro studies, and it is possible to investigate a specific effect of a drug on cells. The time frame of in vivo and in vitro settings is plainly different, and the concentration of oxandrolone used to stimulate cells in the in vitro setting is probably higher than the one used in the clinical trial.[1] Therefore, the results may not be strictly comparable. Although this study demonstrates that oxandrolone is capable of affecting the osteoblast AR and stimulating type I collagen synthesis, regulatory mechanisms that are present only in a complex in vivo situation may account for increased collagen turnover or degradation after synthesis.

Moreover, the delay in the increase of total body bone mineral content reported in the clinical study by Murphy et al[1] may be explained in part by the acute inhibition of bone formation and osteoblast differentiation after a severe burn as previously reported.[3,4] Thus there may have been a lack of osteoblasts, and therefore osteoblast AR, to mediate a response to oxandrolone.

In conclusion, the use of high-dose oxandrolone results in increased nuclear fluorescence of the osteoblast AR, increased osteoblast differentiation markers in cultured osteoblasts and decreased bone marker in cultured osteocyte-like cells in vitro. Oxandrolone may have the ability to directly stimulate bone collagen synthesis, over and above its effect on skeletal loading effected through an increase in lean body mass following long-term treatment. However, longer exposure may no longer be directly anabolic in mature bone cells. This observation may help to explain the variability and confusion regarding androgen actions on the skeleton.

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Poster abstracts from the
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Androgens are known to have pervasive effects on target tissues including muscle and fat, yet the effects on bone remain poorly characterized. To gain insight into the cell types important for mediating androgen action, we constructed and compared two distinct transgenic lines of mice employing different α_1(I)-collagen promoter fragments to control skeletally-targeted androgen receptor (AR) overexpression. The col3.6 AR-transgenic (AR3.6-tg) mice demonstrate AR overexpression throughout the osteoblast lineage including bone marrow stromal cells (BMSC), while in col2.3 (AR2.3-tg) mice AR overexpression is restricted to mature osteoblasts and osteocytes. Complex skeletal analysis using morphological characterization by μCT, dynamic and static histomorphometric analysis, dual-energy x-ray absorptiometry (DXA), biomechanical testing and gene expression studies all indicate that androgen signaling in mature osteoblasts during growth produces a low turnover state, the consequences of which are detrimental to overall matrix quality and bone strength. Unexpectedly, analysis of AR3.6-tg mice also revealed body composition changes in adult males, including reduced fat mass and increased lean mass, an effect not seen in females or in AR2.3-tg mice.

To further examine the effects of enhanced AR signaling on body composition and skeletal quality, an experimental paradigm of protracted hormone ablation followed by steroid replacement was employed. Control (B6D2F2) and AR-tg mice were sham operated or gonadectomized at 3 months of age and the effect of nonaromatizable dihydrotestosterone (DHT) was determined after an 8 week delay, allowing for gonadectomy-induced changes to develop. Following 6 weeks of treatment, the effects of androgen on bone and whole body composition was assessed by DXA. In wild type females, ovariectomy (OVX) resulted in a 13.8 % decrease in BMD while BMC fell 22 % compared to sham (n=14-17). Systemic DHT administration significantly improved BMD and BMC compared to placebo-treated animals (n=14-26). Orchidectomy (ORX) also resulted in reduced BMD and BMC in males (10.5% and 20.3%, respectively)

and 6 weeks of DHT treatment was sufficient to improve or fully restore these deficits in bone mineral. Gonadectomized AR-tg mice also lost bone mineral.

In AR2.3-tg females, BMD and BMC decreased 22.2% and 24.1%, respectively. In contrast to littermate controls, DHT treatment in AR2.3-tg female mice did not improve either bone measure when compared to placebo (n = 3-14). Albeit a small cohort, preliminary results indicate that AR2.3-tg males do not significantly lose BMD or BMC following ORX (n=3-5).

In AR3.6-tg male mice BMD decreased 5.1% while a 15.1 % loss in BMC was observed after ORX.

Steroid loss associated with ageing is known to influence body composition. In addition, in rodent models, gonadectomy has been shown to reduce lean mass and increase fat as a percent of body weight. Consistent with this, our results show that in control males (n = 8-11), lean mass as a percent of body weight was significantly reduced (7.7 %, $p < 0.001$) following ORX while conversely, the percent fat mass increased (7.6 %, $p < 0.001$) compared to sham controls. DHT treatment was beneficial, improving or fully restoring body composition changes induced by ORX compared to placebo. In females (n = 4-10) a similar trend was observed with increased fat (2.2 %) and reduced lean mass (2.3 %) after OVX. Surprisingly, females were resistant to the effects of DHT to restore body composition.

AR2.3-tg mice demonstrated changes in body composition after gonadectomy that mirrored wild types (n=3-9). Again similar to wild-type controls, DHT improved ORX-induced alterations in % fat and % lean mass in AR2.3-tg males but not females.

In contrast to gonadectomized models, DHT treatment in intact wild type mice had a negative impact on bone mineral and body composition in both males and females. Males (n = 14-23) lost 4.4 % lean mass ($p < 0.05$) and increased fat mass by 4.3 % ($p < 0.05$) compared to placebo, with similar but less dramatic changes observed in females (n = 14-26). These results show both males and females increase fat and lose lean mass following gonadectomy. In the bone-targeted AR-overexpression model, enhanced androgen signaling does not influence the response to DHT suggesting that circulating factors derived from bone likely do not play a role in the body composition response to androgen therapy.

Taken together these results indicate that improvements in bone mass with androgen treatment after gonadectomy in wild-type mice are likely mediated through effects on extra-skeletal tissues, not osteoblasts. Consistent with

detrimental effects of enhanced androgen signaling on bone quality, DHT treatment in intact controls significantly reduced bone mass in both males and females. These findings demonstrate that after a sustained hypogonadal period, androgen effectively treats bone loss but that enhanced androgen signaling directly in bone is not anabolic in either male or female adults. The data suggests that targeting androgen response to mature osteoblasts is not beneficial for bone formation, and raise concerns regarding androgen administration or anabolic steroid abuse in healthy individuals in both sexes.

These findings suggest androgen administration has therapeutic advantages in the hypogonadal, but should be approached with caution in healthy adults.
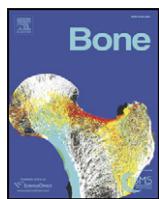
Targeting of androgen receptor in bone reveals a lack of androgen anabolic action and inhibition of osteogenesis
A model for compartment-specific androgen action in the skeleton

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ABSTRACT

Androgens are anabolic hormones that affect many tissues, including bone. However, an anabolic effect of androgen treatment on bone in eugonadal subjects has not been observed and clinical trials have been disappointing. The androgen receptor (AR) mediates biological responses to androgens. In bone tissue, both AR and the estrogen receptor (ER) are expressed. Since androgens can be converted into estrogen, the specific role of the AR in maintenance of skeletal homoeostasis remains controversial. The goal of this study was to use skeletally targeted overexpression of AR in differentiated osteoblasts as a means of elucidating the specific role(s) for AR transactivation in the mature bone compartment. Transgenic mice overexpressing AR under the control of the 2.3-kb α1(I)-collagen promoter fragment showed no difference in body composition, testosterone, or 17ß-estradiol levels. However, transgenic males have reduced serum osteocalcin, CTx and TRAPC5b levels, and a bone phenotype was observed. In cortical bone, high-resolution micro-computed tomography revealed no difference in periosteal perimeter but a significant reduction in cortical bone area due to an enlarged marrow cavity. Endocortical bone formation rate was also significantly inhibited. Biomechanical analyses showed decreased whole bone strength and quality, with significant reductions in all parameters tested. Trabecular morphology was altered, with increased bone volume comprised of more trabeculae that were closer together but not thicker. Expression of genes involved in bone formation and bone resorption was significantly reduced. The consequences of androgen action are compartment-specific; anabolic effects are exhibited exclusively at periosteal surfaces, but in mature osteoblasts androgens inhibited osteogenesis with detrimental effects on matrix quality, bone fragility and whole bone strength. Thus, the present data demonstrate that enhanced androgen signaling targeted to bone results in low bone turnover and inhibition of bone formation by differentiated osteoblasts. These results indicate that direct androgen action in mature osteoblasts is not anabolic, and raise concerns regarding anabolic steroid abuse in the developing skeleton or high-dose treatment in eugonadal adults.

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Introduction

Androgens are steroids that are generally characterized as anabolic hormones, with effects on many tissues including the brain, the immune system, the cardiovasculature, muscle, adipose tissue, liver and bone. Given the large increase in drug sales for testosterone (the major androgen metabolite), estimated at over 500% in the last fifteen years, analysis of the biological consequences of androgen signaling should receive considerable research attention. However, the specific effects of androgen on the skeleton remain poorly characterized and understudied. Since osteoporosis is often coupled with a hypogonadal state in both men and women, sex

steroids are implicated in the maintenance of skeletal health. Although both estrogen and androgen circulate in men and women, albeit at different levels, the influence of each on the remodeling skeleton is distinct [34,55]. Consistent with this, combination therapy with both estrogen and androgen provides an improved response in post-menopausal women compared to estrogen alone [2,47]. Estrogens are thought to act to maintain adult bone mass predominantly through an inhibition of bone resorption by the osteoclast, i.e. as anti-resorptive agents, which protects the skeleton from further loss of bone. Non-aromatizable androgens such as 5α-dihydrotestosterone (DHT), on the other hand, have been proposed as possible bone anabolic agents that increase bone formation and bone mass [30,35].

In support of an anabolic effect of androgen on the skeleton, free testosterone concentrations have been shown to correlate with bone mineral density (BMD) in elderly men [56], however testosterone levels

also correlated with muscle mass and strength. Testosterone treatment is effective at ameliorating bone loss during aging, but only in men with low testosterone levels [8,61]. Conversely, men undergoing androgen deprivation therapy for prostate cancer show significantly decreased BMD [49] and an increase in clinical fractures [32]. During growth, there are gender differences in skeletal morphology that develop with puberty particularly in cortical bone, with radial expansion that is predominantly observed in boys [31]. Combined, these findings suggest that androgens serve important functions to both maintain bone mass in the adult and to influence the growing, modeling skeleton (see[63]).

Nevertheless, a controversy exists regarding the consequences and/ or importance of androgen signaling on skeletal homeostasis. Whether the observed effects of circulating testosterone are due to direct effects on bone is complicated by the fact that androgens influence a variety of tissues known to be associated with bone health, most importantly muscle. Nonetheless, bone is a direct target tissue with respect to androgen action. AR is expressed in the cell types required for skeletal formation and homeostasis, including mesenchymal stromal precursors [4], osteoblasts [1], osteocytes [1,64] and osteoclasts [57]. An additional complication for interpretation of the direct effects of testosterone results from the consequences of its metabolism. Since testosterone serves as the substrate for estradiol synthesis through the action of the enzyme aromatase, systemic testosterone may have effects mediated predominantly or exclusively through activation of estrogen receptor (ER) signaling. Therefore, a specific role for AR signaling cannot be inferred with simple testosterone therapy.

In addition, not all of the studies examining the association of testosterone levels with BMD in adults have actually shown a positive correlation. In general, correlations between bone mass and serum androgen concentrations in adult men have been either weak or insignificant [17,42,48]. Furthermore, many of the various clinical trials examining androgen therapy have been unable to demonstrate robust effects on bone mass, including treatment with anabolic steroids [11]. In most studies that do show an increase in BMD, the most marked improvement is observed only in men with the lowest testosterone levels [61]. Notably, an anabolic effect of androgen treatment on bone in eugonadal men (or in women) has not been observed, in contrast to known anabolic dose-dependent effects to increase muscle mass [5]. For these reasons and because of concerns about safety, androgen replacement even in hypogonadal men remains a controversial issue [20]. Given the modest therapeutic benefit observed with androgen therapy [33], speculation has arisen that a portion of the positive effect of androgens on bone mass may be mediated indirectly through known effects to increase muscle mass and strength. An increase in lean mass would have beneficial effects on BMD though biomechanical linkage and skeletal adaptation. Consistent with this suggestion, Murphy et al [39] have shown that administration of the synthetic anabolic androgen oxandrolone to severely burned children increases lean body mass three to six months before an increase in bone mineral content is observed.

Not surprisingly given the complex nature of bone tissue, systemic androgen administration has shown distinct responses in different skeletal compartments, i.e., cortical, trabecular (cancellous) or intramembranous bone. In hypogonadal settings, a beneficial response to androgen therapy is observed in the trabecular compartment, the more active surface in bone, with an increase in bone mass. However, this relative increase in bone mass occurs with suppression of bone resorption, with micro-architectural changes demonstrating an increase in trabecular number but not thickness. For example, histomorphometric analysis of androgen replacement in hypogonadal male mice has shown that AR activation preserves the number of trabeculae but does not maintain thickness, volumetric density or mechanical strength [38]. Notably, these studies also demonstrated that the bone-sparing effect of AR activation is distinct from the bone-sparing effect of ERα. In addition, androgen appears to play an important role in intramembranous bone formation [16]. Finally, there are reports of increased cortical bone mass, as a consequence of increased bone width and surface periosteal expansion (see[59,63]). High-dose testosterone therapy over 2 years in (genetic female) female-to-male transsexuals resulted in increased areal BMD at the femoral neck, in a setting where estradiol declined to post-menopausal levels [54]. In men with constitutional delay of puberty, impaired periosteal expansion is observed [68]. Taken together, these reports indicate that androgens in vivo act to maintain trabecular bone mass through inhibition of osteoclast activity and to expand cortical bone at the periosteal surface. While these findings argue that androgen positively affects cortical bone at the periosteal surface (see[67]), what is lacking is clear documentation of an anabolic effect to increase bone formation in mature osteoblasts and osteocytes. Thus, the direct consequences of androgen action on differentiated osteoblasts in vivo remain unclear, and mechanisms underlying potential positive outcomes on bone formation and bone mass remain uncharacterized.

Concentrations of estrogen and androgen receptors vary during osteoblast differentiation, with AR levels highest in mature osteoblasts and osteocytes [64]. Since osteocytes are the most abundant cell type in bone [50], these cells are likely an important target cell for androgen action, and may represent a central mediator for skeletal responses to testosterone therapy in vivo. The goal of this study was to use skeletally targeted overexpression of AR as a means of elucidating the specific role(s) for AR transactivation in the mature differentiated osteoblast. AR over-expression was targeted by the col2.3 promoter and was chosen for several reasons: the skeletal expression patterns for this promoter are bone-selective and well-characterized (see[24–26,36,37]); with strong col2.3 promoter activity in differentiated osteoblasts/osteocytes and mineralizing nodules [25,26,36] but not in osteoclasts [7]. Promoter activity varies in different bone compartments. In intramembraneous bone, strong expression was seen in cells at osteogenic fronts of parietal bones but not in the suture [36]. In long bones, strong transgene expression was observed in most osteoblasts on endocortical surfaces, and in a large proportion of osteocytes in femurs throughout cortical bone but with no expression in periosteal fibroblasts [25,26]. In the trabecular area of metaphyseal bone, strong expression was observed at all developmental stages [26].

A distinct advantage of employing a transgenic model is the enhancement of androgen signaling in a specific target in vivo, e.g., mature osteoblasts and osteocytes, as a consequence of increased AR abundance. The AR2.3-transgenic model allows for characterization of skeletal responses in the absence of changes in circulating hormone (testosterone or 17b-estradiol) that occurs with global genetic manipulations, to take advantage of increased sensitivity to androgen in distinct skeletal sites for analysis of compartment-specific effects. At the same time, manipulation of androgen action through AR overexpression, rather than systemic administration, gonadectomy or global knockout, excludes effects that would occur at other androgen target tissues in vivo including muscle and fat. Here we describe the skeletal consequences of enhanced androgen signaling that is restricted to mature osteoblasts and osteocytes, employing the 2.3-kb type I collagen promoter to control AR overexpression.

Materials and methods

Cloning of expression plasmids

The pBR327-based plasmid col2.3-ßgal-ClaPa contains the basic rat collagen Iα1 promoter sequence − 2293 to + 115 (provided by Dr. David Rowe, University of Connecticut Health Center), which served as the starting vector. BamHI sites were added to the rat AR cDNA (provided by Dr. Shutsung Liao, University of Chicago) with PCR primers. The PCR product was T/A cloned in pCR 2.1-TOPO vector (Invitrogen Corp., Carlsbad, CA, USA). Finally the BamHI-rAR fragment was cloned into the BamHI site in the col2.3-ßgal-ClaPa (after removal of the ßgal cDNA sequences), to give the expression construct referred to as the AR2.3-transgene. The correct sequence and orientation of the AR insert was verified by direct DNA sequencing.

Generation of AR2.3-transgenic mice

AR2.3-transgenic mice were produced using standard technology by the Oregon Health and Science University (OHSU) Transgenic Mouse Facility, following methodology previously described [67]. Embryos were obtained from matings of C57BL/6 males × DBA/2J

females (B6D2F1). Founder mice were identified by PCR genotyping and mated with B6D2F1 (Jackson Labs, Bar Harbor, ME, USA) to produce F2 litters. Transgenic mice were healthy and transmitted the transgene at the expected frequency. The generation and use of transgenic mice were performed according to institutional, local, state, federal and NIH guidelines for the use of animals in research under an Institutional Animal Use and Care Committee-approved protocol.

Animals

AR2.3-transgenic mice were bred to B6D2F1 mice (Jackson Labs); both genders were employed. The mice had free access to tap water and were fed a diet containing 1.14% calcium, 0.8% phosphorous, 2200 IU/kg vitamin D3, 6.2% fat and 25% protein (Purina PMI Nutrition International, St. Louis, MO, USA). All animals were weighed weekly, and body length (nose to rump) was determined at monthly intervals over six months ($n = 4$–5). For analysis, the animals were sacrificed under CO_2 narcosis by decapitation. The right femur was used for measurement of cortical and trabecular volumetric density and geometry by ex vivo micro-computed tomography (μCT), followed by destructive analysis of whole bone biomechanical properties. The left femur was used for dynamic histomorphometric analysis. The length of the femur was measured from the femoral head to the distal condyles. In addition, a variety of tissues/organs were collected for RNA isolation or histological and immunohistochemical analyses. For RNA isolation, tibia was cleaned of muscle tissue and aseptically dissected. After removal of the epiphyseal area, marrow was briefly flushed with sterile phosphate buffered saline and the bone was frozen in liquid nitrogen and stored at −80 °C until RNA isolation as described below.

Primary calvarial osteoblast culture and western blot analysis

Primary osteoblastic cells were isolated after collagenase digestion from fetal calvaria from both wild-type and AR2.3-transgenic fetal mice. Calvariae were isolated from 3–6 day old mice after genotyping and subjected to four sequential 15-min digestions in a mixture containing 0.05% trypsin and 0.1% collagenase-P at 37 °C. Cell fractions 2–4 were pooled and plated at 8000 cells/cm^2 in MEM supplemented with 10% FBS. Beginning at confluence around day 7, cultures were switched to differentiation medium in phenol-red free BGJb (Fitton-Jackson modification) containing 50 μg/ml ascorbic acid. From day 14 on, 5 mM ß-glycerophosphate was added to the differentiation media. Whole cell lysates were prepared with lysis buffer. Equal amounts of cell extract were electrophoresed on a 10% SDS-polyacrylamide gel, and the separated proteins were transferred to an Immobilon-P polyvinylidene-difluoride transfer membrane (Millipore, Bedford, MA, USA). AR abundance was determined by immunoblotting with polyclonal rabbit AR antibodies (ARN-20) purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and used at 1:300 dilution. The α-tubulin antibody (T9026) was a mouse monoclonal antibody purchased from Sigma and was used at 1:1000 dilution. Bound antibodies were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) on Kodak X-AR5 autoradiographic film. The analysis of α-tubulin was used for protein loading control.

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted and the concentration was measured at 260 nm using a spectrophotometer, with purity assessed by the $A_{260/280}$ ratio. RNA integrity was confirmed on a 1% agarose gel after SYBR Gold staining (Invitrogen Corp) and qRT-PCR was performed as previously described [19]. Intron-spanning primers for tibial RNA analysis were purchased pre-designed from Qiagen (Valencia, CA, USA). Transgene-specific RT primers were forward 5′-GCATGAGCCGAAGCTAAC-3′ and reverse 5′-GAACGCTCCTCGATAGGTCTTG-3′ designed using Oligo Software (Molecular Biology Insights, Inc. Cascade CO, USA), and specifically amplified colAR using sites in the collagen untranslated region and AR near to those used for genotyping. Fold regulation was determined by normalizing all values to the mean of the value in calvaria.

Serum biochemistry and hormone analysis

Serum specimens from 2-month-old female and male mice of both genotypes were collected and stored at −20 °C until analysis was performed ($n = 6$–17). Blood samples were obtained under anesthesia by cardiac puncture. Serum analysis was as previously described [67], with 17ß-estradiol measured by radioimmunoassay (RIA) using Immuchem Double Antibody 17ß-Estradiol RIA (ICN Biomedicals Inc., Costa Mesa, CA, USA); testosterone measured by enzyme linked immunoassay from Diagnostic Automation Inc. (Calabasas, CA, USA); osteocalcin quantitated by ELISA (Biomedical Technologies Inc., Stoughton, MA, USA); and OPG determined by immunoassay kit (R&D systems, Minneapolis, MN, USA). Indices of bone resorption in vivo were quantitated using the serum biochemical marker C-terminal telopeptide of collagen (CTx) analyzed by a RatLaps ELISA kit (Nordic Bioscience Diagnostics A/S, Herlev, Denmark). Assays were performed using 20 μl serum aliquots in duplicate following the manufacture's recommendations. Intraassay variation was 5.6%, and interassay variation was 10.5%. Serum TRAPC5b activity was determined using MouseBoneTRAP Assay (Immunodiagnostic Systems Inc., Fountain Hills, AZ, USA) using 25 μl serum aliquots in duplicate following the manufacturer's protocol.

Histochemical analysis of calvaria

Immunohistochemical analysis was performed on representative calvaria from 2-month-old mice. Calvaria were fixed, decalcificied in Immunocal (Decal Corp., Tallman, NY, USA), and sections were processed by dehydration, paraffin infiltration and embedding. Tissue sections (5–6 μm) were cut, processed and subjected to immunohistochemical staining after incubation in a primary antibody directed against AR N-terminus (ab3510, 4 μg/ml; Abcam Inc, Cambridge, MA, USA) at 4 °C overnight. For AR detection, sections were incubated for 1 h in a biotinylated goat antirabbit secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA). Following rinses, sections were incubated for 60 min in avidin-biotin complex (1: 1000; Vectastain Elite; Vector Laboratories). After 30 min of rinsing, sections were incubated for 10 min with a diaminobenzidine (DAB) solution (0.05% DAB) activated by 0.001% hydrogen peroxide. Slides were counterstained with hematoxylin.

Dynamic histomorphometry

Bone formation and resorption during the last week of growth was assessed by dynamic histomorphometric measures after fluorochrome labeling ($n = 8$–20 males; 10–15 females). Prior to sacrifice, 2-month-old mice received two fluorochrome labels by intraperitoneal (ip) injection [oxytetracycline hydrochloride (Sigma, St. Louis, MO, USA) at 30 mg/kg and calcein green (Sigma) at 10 mg/kg], given 10 days and 3 days before death, respectively. Left femora were dissected and processed non-decalcified for plastic embedding as previously described [23]. Cross-sections (100 μm) through mid-diaphysis were prepared using a diamond-wafering saw (Buhler, Lake Bluff, IL, USA), then polished to a thickness of 30 μm. Sections were left unstained and dynamic histomorphometry was carried out using a light/epifluorescent microscope and a semiautomatic image analysis system (OsteoMetrics, Inc., Decatur, GA USA). Standard measures of bone formation and resorption were determined for both the periosteum and endocortex, including mineral apposition rate (MAR, μm/day), mineralizing perimeter ([dL.Pm+sL.Pm/2]/B.Pm, %), bone formation rate (BRF/B.Pm, μm^2/μm/day), and eroded perimeter (%). Bone formation rate (perimeter referent) was calculated by multiplying mineralizing perimeter by mineral apposition rate. In addition, the cross-sectional area and cortical area were measured on cortical cross-sections and cortical thickness calculated. The terminology and units used were those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research [44].

Bone morphology and microstructure

The morphological consequences of increased AR expression in osteoblastic/osteocytic cells in AR2.3-transgenic animals were evaluated in 2-month-old male and female mice by dual energy X-ray absorptiometry (DXA) and quantitative μCT. Areal BMD, bone mineral content (BMC) and body composition was measured by whole body DXA using a mouse densitometer (PIXImus2, Lunar, Madison WI, USA). Right femurs from each genotype ($n = 10$–21 males; 13–19 females) were examined for diaphyseal cross-sectional morphology and tissue mineral density (TMD) using an eXplore Locus SP Pre-Clinical Specimen MicroComputed Tomography system (GE Medical Systems, London, Ontario) as described previously [22]. Area measures were body weight adjusted to reduce variability. Three-dimensional images of the entire femur were obtained at an 8.7 μm voxel size and individually thresholded using a standard segmentation algorithm [43]. A 3 mm region of the reconstructed mid-diaphysis, corresponding to the typical failure region for 4-point bending (see below), was examined. Determination of cross-sectional morphology was performed using custom analysis program (MathWorks, v. 6.5; The MathWorks, Inc., Natick MA, USA) [22]. Trabecular morphometry of the distal metaphysis, including bone volume fraction (BV/TV) and trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp), was assessed using Microview Advanced Bone Analysis (GE Medical Systems, v. 1.23).

Images from the μCT analysis were also used to quantify TMD as described previously [22]. TMD is the average mineral value of the bone voxels alone, expressed in hydroxyapatite (HA) density equivalents. TMD was calculated by converting the grayscale output of bone voxels in Hounsfield units (HU) to mineral values (mg/cc of HA) through the use of a calibration phantom hydroxyapatite (SB3: Gamex RMI, Middleton, WI, USA) [TMD = average bone voxel HU / average HA phantom HU * 1130 mg/cm^3 (HA physical density)] [22]. The same calibration phantom was used for all scans to normalize mineral density measurements and to account for possible variability among scan sessions.

Mechanical testing

Following DXA and μCT analysis, the right femurs were subjected to destructive testing to establish whole bone mechanical properties. Femurs were loaded to failure in 4-point bending. All whole bone-bending tests were conducted by loading the femurs in the posterior to anterior direction. The load-deflection curves were analyzed for stiffness (the slope of the initial portion of the curve), maximum load, post-yield deflection (PYD), and work-to-failure as described previously [23]. Stiffness and maximum load were adjusted for body weight.

Statistical analysis

All data were analyzed using Prism software v4 (GraphPad Software, Inc., San Diego, CA, USA). Significance of difference between wild-type and AR2.3-transgenic mice was assessed by an unpaired two-tailed t-test using Welch's correction. Body lengths and weights were analyzed by repeated measures two-way ANOVA for the effects of gender and genotype. All data are shown as mean ± standard error of the mean (SEM).

Results

Generation of transgenic mice with enhanced androgen signaling in mature osteoblasts and osteocytes

Confusion exists regarding the *in vivo* action of androgens in bone due to metabolism to estrogen, because androgen influences many tissues in the body, and many months of treatment are required to observe improvement in BMD. The AR2.3-transgenic animal model was created to determine the specific physiologic relevance of androgen action in the mature osteoblast/osteocyte population in bone, through tissue-specific overexpression of AR. This line is distinct from our previously described AR3.6-transgenic model, with AR overexpression in stromal precursors, periosteal fibroblasts and throughout the osteoblast lineage directed by the col3.6 promoter [67]. A transgene cassette (AR2.3) was cloned as described in Materials and methods and AR2.3-transgenic mice were created following standard procedures. Positive founders were identified by PCR genotyping and were bred to wild-type B6D2F1 mice; two AR2.3-transgenic lines (lines 219 and 223) derived from independent founders have been retained. Southern analysis confirmed a single insertion site for the AR2.3-transgene (data not shown). Table 1 lists the qRT-PCR analysis of expression of the AR2.3-transgene in various tissues, showing the expected bone targeting with highest levels in calvaria but ~100–3000-fold lower levels in muscle, skin, heart, intestine, kidney, liver, lung and spleen.

Phenotype in AR2.3-transgenic mice with bone-targeted AR overexpression

To begin to characterize the phenotype of AR2.3-transgenic mice, we first determined body weight gain and nose–rump length over a 6-month period. At birth, animals were indistinguishable and as the mice aged, AR2.3-transgenic males and females gained length and weight similar to wild-type controls (Figs. 1a, b). Body composition and bone density were evaluated by DXA at 2 months in male and female AR2.3-transgenic mice and wild-type littermate controls. While systemic androgen treatment is known to affect body compositional changes, no difference was noted in either lean mass (Fig. 1c) or fat mass (Fig. 1d) in either males or females, consistent with skeletal targeting of the AR transgene. In addition, areal BMD (Fig. 1e) and BMC (Fig. 1f) were not significantly different in males or females, indicating a lack of effect on periosteal surfaces as expected.

AR2.3-transgenic mice were next evaluated for serum biochemistry and hormone levels at 2 months of age. As expected with bone targeting of AR overexpression, serum testosterone and estradiol levels were not significantly different between littermate controls and transgenic animals in either sex (Figs. 2a, b). There were also no significant

Table 1
Analysis of transgene expression in a variety of tissues from AR2.3-transgenic mice

Tissue	AR2.3-tg level	Fold difference
Calvaria	1.0000±0.2373	n.a.
Thymus	0.0066±0.0011	−152
Lung	0.0054±0.0005	−185
Heart	0.0047±0.0019	−213
Kidney	0.0044±0.0006	−227
Fat	0.0027±0.0007	−370
Spleen	0.0025±0.0004	−400
Muscle	0.0006±0.0000	−1667
Skin	0.0006±0.0002	−1667
Ear	0.0004±0.0001	−2500
Liver	0.0004±0.0002	−2500
Tendon	0.0003±0.0000	−3333
Intestine	0.0000±0.0000	n.d.

Tissues listed were harvested from male AR2.3-transgenic mice and total RNA was isolated ($n=5$). Expression of the transgene was evaluated by real-time qRT-PCR analysis after normalization to the total RNA concentration using RiboGreen [19]. Data are expressed relative to the expression level in calvaria as mean±SEM. n.a., not applicable; n.d., not detectable.

differences in serum calcium levels between transgenic mice and littermate controls (Fig. 2c). Interestingly, there was a significant ~50% decrease in serum osteocalcin levels in male AR2.3-transgenic animals ($P<0.01$, Fig. 2d), but not in females. Serum OPG showed little difference between genotypes (Fig. 2e), but bone resorption markers TRAPC5b and CTx (that mostly reflect cortical bone properties) were significantly reduced in male AR2.3-transgenic mice ($P<0.05$, Figs. 2f, g).

To further evaluate the phenotype of AR2.3-transgenic mice, we characterized AR protein expression *in vivo* by immunohistochemical analysis in calvarial sections from both sexes (Fig. 2h). The antibody used does not distinguish between endogenous AR and the product of the AR2.3-transgene, so immunostaining represents combined levels for both proteins. AR expression is seen in osteocytes in wild-type mice (brown color combined with blue color from hematoxylin counterstaining), in sections from the middle of the calvaria (bottom panels for each sex). Consistent with increased AR2.3-transgene expression, robust AR expression is seen in osteocytes in both male and female transgenic mice (bottom panels for each sex). AR is also seen in differentiating osteoblasts at the innermost layer at the bone surface (upper panels for each sex). Thus, increased AR expression in osteocytes and the most differentiated osteoblasts is seen in both male and female transgenic animals, consistent with previous characterization of promoter activity [25,26]. There was no notable difference between the sexes or between independent families (data not shown). Morphological changes were evaluated by H&E staining (data not shown); there was no difference in calvarial width between wild-type and AR2.3-transgenic mice of either gender, nor between the independent AR2.3-transgenic families 219 and 223. In contrast, calvaria from male AR3.6-transgenic mice, with AR overexpression in periosteal fibroblasts and throughout the lineage, demonstrated substantial calvarial thickening [67]. Finally, we examined AR levels by Western analysis in *ex vivo* studies using primary calvarial osteoblast cultures derived from wild-type *vs.* transgenic mice. As shown in Fig. 2i, increased AR levels are seen in mature osteoblasts/osteocytes at day 30 in differentiating cultures from transgenic mice.

Enhanced androgen signaling results in altered bone morphology and reduced cortical area in male transgenic mice

Overall cortical bone morphology and femoral structure were quantified from high-resolution μCT images. Measures obtained for morphological assessment from μCT analysis are described in Fig. 3a. No effect of AR2.3-transgene expression on total cross-sectional area or surface periosteal perimeter was observed in either males or females (Figs. 3b, e). However, marrow cavity area was significantly increased in transgenic males (*i.e.*, reduced infilling occurred; $P<0.01$, Fig. 3c). Given no compensatory changes in the periosteal layer, this inhibition resulted in a modest but significant reduction in cortical bone area in male transgenic mice ($P<0.05$, Fig. 3d). Thus, enhanced AR signaling in mature osteoblasts has significant inhibitory effects on overall femoral cortical bone area. This morphological difference at the diaphysis was not observed in female transgenic mice. We also evaluated polar moment of inertia and tissue mineral density (TMD) at the mid-diaphysis. While there was no significant effect on polar moment of inertia (Fig. 3f), transgenic males show a significant reduction in TMD ($P<0.001$; Fig. 3g).

Reduced endocortical bone formation with bone-targeted AR overexpression

Because of the changes observed with the μCT analysis in cortical bone area in transgenic males, dynamic histomorphometric analysis was carried out at the femoral diaphysis using fluorescent imaging. Fluorochromes were administered (oxytetracycline followed by calcein) to label new mineral deposition. Fig. 3h shows patterns of bone formation in images of fluorochrome labeling from femoral cross-sections. The AR2.3-transgenic males (upper panel) show a

Fig. 1. Weight changes and body composition analysis in AR2.3-transgenic mice. Body weight and nose–rump-length determinations were carried out weekly or monthly, respectively, over six months in both genders in both wild-type (wt) and AR2.3-transgenic (AR2.3-tg) mice. a. Weight gain in growing male (left) and female (right) mice. Analysis for the effects of time and genotype by repeated measures two-way ANOVA in males revealed an extremely significant effect of time ($F=218.36$; $P<0.0001$) but not genotype, and with no interaction; females were similar ($F=114.80$; $P<0.0001$). b. Nose–rump length in male (left) and female (right) mice. Analysis by repeated measures in males revealed an extremely significant effect of time ($F=228.54$; $P<0.0001$) and an effect of genotype ($F=15.87$; $P<0.01$) with no interaction; females only showed an effect of time ($F=149.48$; $P<0.0001$). Data is shown as mean±SEM, $n=4$–5. DXA was performed on 6-month-old AR2.3-transgenic and littermate control mice to assess bone mineral, lean mass and fat mass. c. Lean mass adjusted for total tissue mass. d. Fat mass adjusted for total tissue mass. e. Areal BMD (minus head). f. BMC. Values are expressed as mean±SEM, $n=4$–10.

dramatic lack of labeling at the endocortical surface compared with wild-type controls (lower panel). Consistent with these fluorescent images, quantitative dynamic histomorphometric analysis revealed inhibitory responses at the endocortex in transgenic males, with strong inhibition (~70%) of BFR/B.Pm ($P<0.001$; Fig. 3i) and ~50% reduction in MAR, the latter being a measure of osteoblast vigor ($P<0.001$; Fig. 3j). Eroded perimeter was reduced at the periosteum

($P<0.001$; Fig. 3k), while mineralizing perimeter (M.Pm/B.Pm) showed inhibition at the endocortex ($P<0.01$) but stimulation at the periosteum ($P<0.01$) in Fig. 3l. The modest increase in periosteal activity seen does not parallel a change in cortical bone morphology characterized by μCT analysis, likely since the labeling is representative of mineralization patterns only for the period of time that the labels are present.
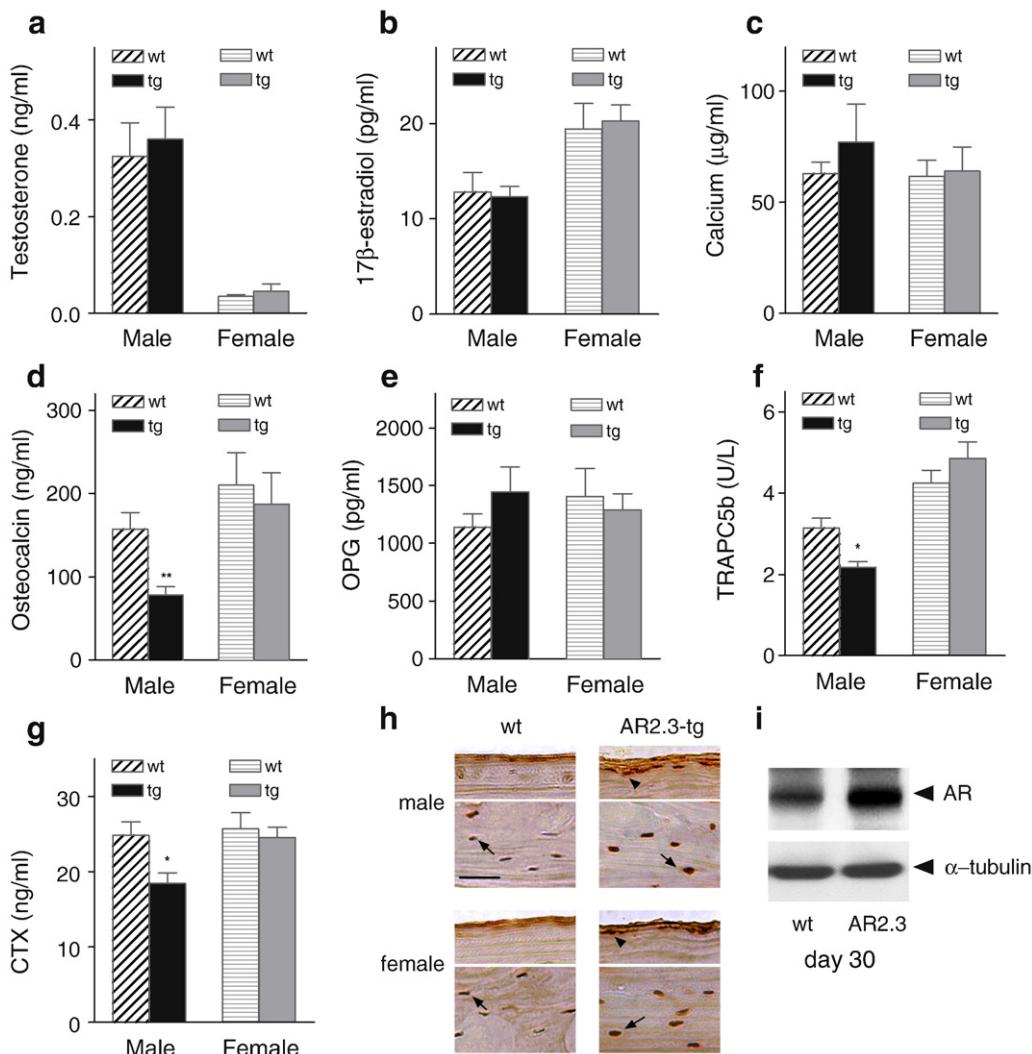
Fig. 2. Phenotypic characterization of serum markers and AR overexpression in AR2.3-transgenic animals. Comparisons were performed between wild-type littermate control (wt) and AR2.3-transgenic (AR2.3-tg) animals. Serum from 2-month-old mice was analyzed to determine levels of hormones and markers of calcium metabolism. Assays were performed in duplicate by RIA for 17ß-estradiol or EIA for testosterone, OPG and intact mouse osteocalcin, and for calcium by the colorimetric cresolphthalein-binding method. a. Testosterone. b. 17ß-estradiol. c. Calcium. There were no statistical differences between the genotypes for 17ß-estradiol, testosterone or calcium levels. Values are expressed as mean±SEM, $n=6$–17. d. Osteocalcin. e. OPG. f. TRAPC5b. g. CTx. Osteocalcin, TRAPC5b and CTx were all significantly reduced in male AR2.3-transgenic mice. Values are expressed as mean±SEM, $n=3$–8. $*P<0.05$; $**P<0.01$ (vs. gender-appropriate wild-type control). h. Immunohistochemical analysis of AR levels in calvaria isolated from 2-month-old mice. Sections were subjected to immunohistochemical staining after demineralization and paraffin embedding. For each sex, the top panel represents a section at the calvarial surface to focus on osteoblasts (arrowheads) while the bottom panel represents a section through the center of the bone to show osteocytes (arrow). Representative sections are shown. AR abundance was visualized with rabbit polyclonal antisera for male and female mice from wt and AR2.3-tg mice. AR is brown and the nucleus is purple after DAB incubation and counterstaining with hematoxylin. Scale bar=50 μm. i. AR levels by Western blot analysis during ex vivo differentiation of calvarial osteoblasts derived by collagenase digestion from wild-type or AR2.3-transgenic mice. Analysis was performed at day 30 in mineralizing cultures. Control for loading was characterized by α-tubulin levels.

Ability to resist fracture is impaired in male AR2.3-transgenic mice

To analyze whole bone biomechanical and failure properties, femurs from 2-month-old wild-type and AR2.3-transgenic animals were loaded to failure in 4-point bending at 0.05 mm/s. Although overall geometry of the femur showed no obvious differences between wild-type and transgenic mice (Fig. 4a), failure properties were significantly impaired. Diaphyseal strength assessed as both maximum load ($P<0.05$, Fig. 4b) and stiffness ($P<0.05$, Fig. 4c) was decreased slightly by about 10% in male transgenics (with no change in females), consistent with the decreased cortical bone area (see Fig. 3c). However, a dramatic impairment was seen in male transgenic bones in their ability to resist fracture. They were more brittle (less ductile), with an approximately 40% decrease in post-yield deflection ($P<0.05$, Fig. 4d). Work-to-failure was reduced by nearly 30% compared to wild-type control bones ($P<0.05$, Fig. 4e). Male

AR2.3-transgenic mice in this cohort showed no difference in femoral length or weight (Figs. 4f, g).

AR overexpression in bone results in increased trabecular bone volume in male transgenic mice

We also used μCT analysis to evaluate the consequences of AR overexpression on the trabecular bone compartment. Visualization of trabecular bone in the metaphysis after manual subtraction of the cortical shell shows an increase in trabecular bone volume in male AR2.3-transgenic mice (Fig. 5a). To better characterize trabecular micro-anatomy and architecture, static histomorphometric analysis was performed from images of the metaphyseal trabecular region (Figs. 5b–e). Male AR2.3-transgenic mice showed an ~35% increase in trabecular bone volume as a percent of tissue volume (BV/TV; $P<0.05$; Fig. 5b), consistent with the μCT image. The increase in trabecular
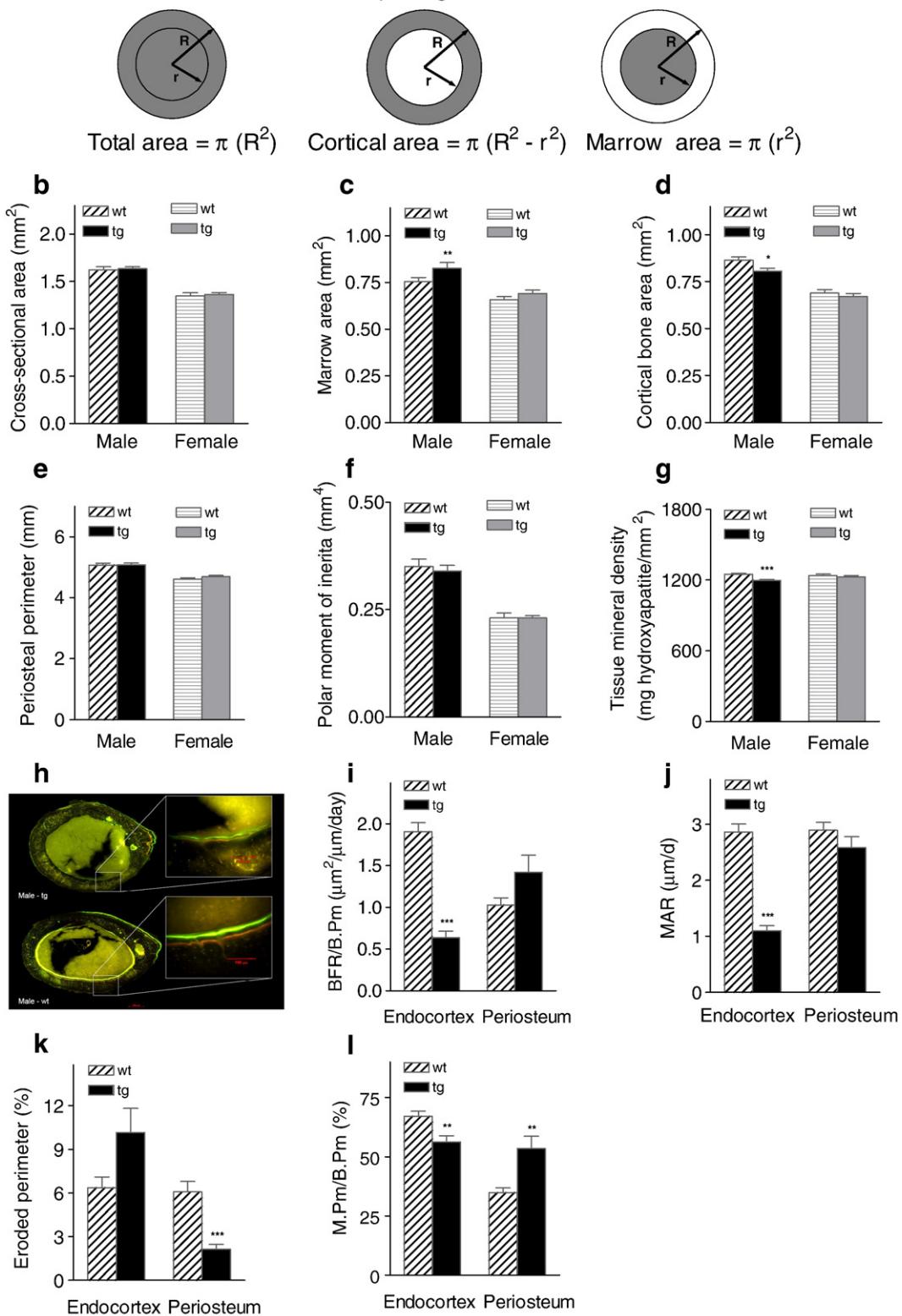
Fig. 3. Cortical morphology, structural analysis and bone formation rates in AR2.3-transgenic mice. Femurs were isolated from 2-month-old male and female wild-type (wt) or AR2.3-transgenic mice (tg) and subjected to high-resolution μCT imaging at mid-diaphysis. a. Parameters for morphological characterization by μCT. b. Total cross-sectional area. c. Marrow cavity area. d. Cortical bone area. e. Periosteal perimeter. f. Polar moment of inertia. g. Tissue mineral density. Values are shown as mean±SEM, $n=10$–21 males; 13–19 females. Differences between genotypes were determined by Student's unpaired t-test with Welch's correction. *$P<0.05$; **$P<0.01$; ***$P<0.001$ (vs. gender-appropriate wt controls). For dynamic histomorphometric analysis, male femurs were sectioned at the mid-diaphysis; rates were determined at both the endocortex and periosteum. h. Fluorescent images of femur after double-label administration. Representative photomicrographs are shown with higher power insets demonstrating labeling on the endocortex. Bands were photographed at comparable anatomic positions for each bone. i. Bone formation rate (BFR). j. Mineral apposition rate (MAR). k. Percent eroded perimeter. l. Percent mineralizing perimeter (M.Pm/B.Pm×100). Values are shown as mean±SEM; $n=8$–20 males; 10–15 females. **$P<0.01$; ***$P<0.001$ (vs. wt controls). Scale bar=200 μm in figure; scale bar=100 μm in insets as indicated.
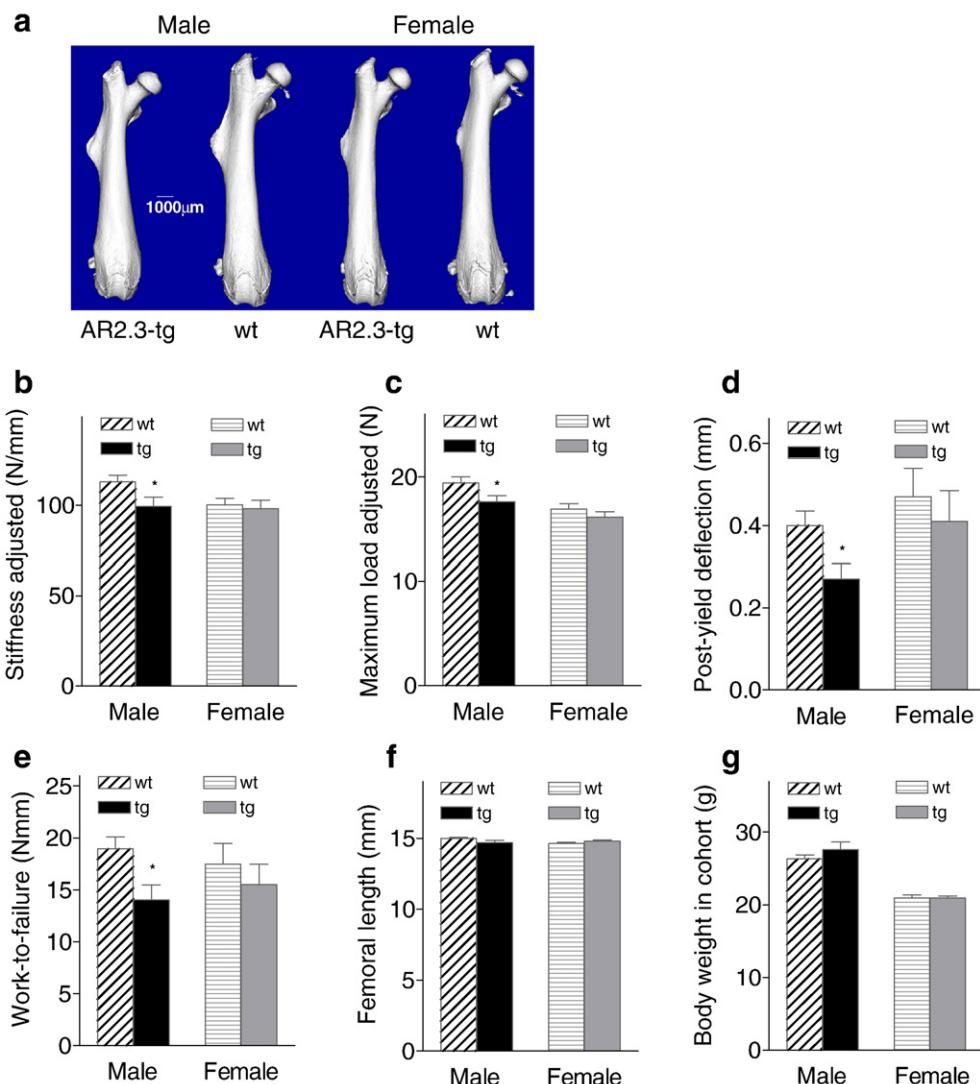
Fig. 4. Whole bone strength and failure properties determined from biomechanical analyses. Femurs from 2-month-old male and female wild-type (wt) and AR2.3-transgenic (tg) mice were loaded to failure in 4-point bending analysis. Stiffness, maximum load, and post-yield deflection were calculated from the load-deflection curves. Stiffness and maximum load are adjusted for body weight differences. a. Whole bone morphology from μCT imaging. b. Stiffness adjusted. c. Maximum load adjusted. d. Post-yield deflection. e. Work-to-failure. f. Femoral length. g. Body weight in cohort. Whole bone biomechanical properties are shown as mean±SEM, $n = 10$–21 males; 13–19 females. Differences between genotypes were determined by Student's unpaired t-test with Welch's correction. *$P < 0.05$ (vs. gender-appropriate wt controls).

bone volume was associated with an ~25% increase in trabecular number (Tb.N; $P < 0.01$; Fig. 5c), with no effect on trabecular thickness (Tb.Th; Fig. 5d), and thus an ~30% decrease in spacing (Tb.Sp; $P < 0.01$; Fig. 5e).

Enhanced androgen signaling in mature osteoblasts leads to reduced expression of molecular markers of bone formation and osteoclast activation in cortical bone

Lastly, we analyzed gene expression in long bone from wild-type and AR2.3-transgenic mice of both genders. Differences in gene expression in RNA isolated from tibial mid-diaphysis for sets of genes that play a significant role in either bone formation or bone resorption (Fig. 6). Osteoblastic marker genes evaluated were cyclin D1, osterix, type I collagen (col), osteocalcin (OC) and sclerostin (SOST). Levels of osteoblastic marker genes are listed in an order reflecting their temporal expression patterns during osteoblast differentiation, *e.g.*, osteocalcin is expressed late in osteoblast differentiation. Significant reductions were noted in transgenic males in levels of osterix, collagen, osteocalcin and SOST (Fig. 6a, left panel). The reduction in

osteocalcin gene expression mirrors the reduction in serum concentrations (Fig. 2d). Interestingly, expression of osteoblastic markers in mid-diaphyseal tissue from male transgenic mice was inversely correlated with the differentiation stage, with increased inhibition for genes representative of more mature differentiated osteoblasts, suggesting alteration of the organic matrix consistent with reduced osteoblast vigor shown in Fig. 3j. For example, SOST is produced by mature osteocytes [46], and SOST mRNA levels were significantly inhibited in male transgenic bones. This pattern mirrors the expected increase in col2.3 promoter activity during osteoblast differentiation [12]. Osteoclastic marker genes analyzed were OPG, receptor activator of NF-κB ligand (RANKL), tartrate-resistant acid phosphatase (TRAP), cathepsin K (CatK) and calcitonin receptor (CTR). Similar to the osteoblastic marker genes, inhibition of gene expression was also observed in osteoclastic marker genes CTR, CatK, and TRAP again only in male AR2.3-transgenic mice (Fig. 6a, right panel). The reduction osteoclastic gene expression is consistent with reduced serum levels of bone resorption markers (TRACP5b and CTx; Figs. 2e, f). This qRT-PCR analysis represents changes observed in cortical bone, and may not reflect similar changes in trabecular bone. Consistent with the lack
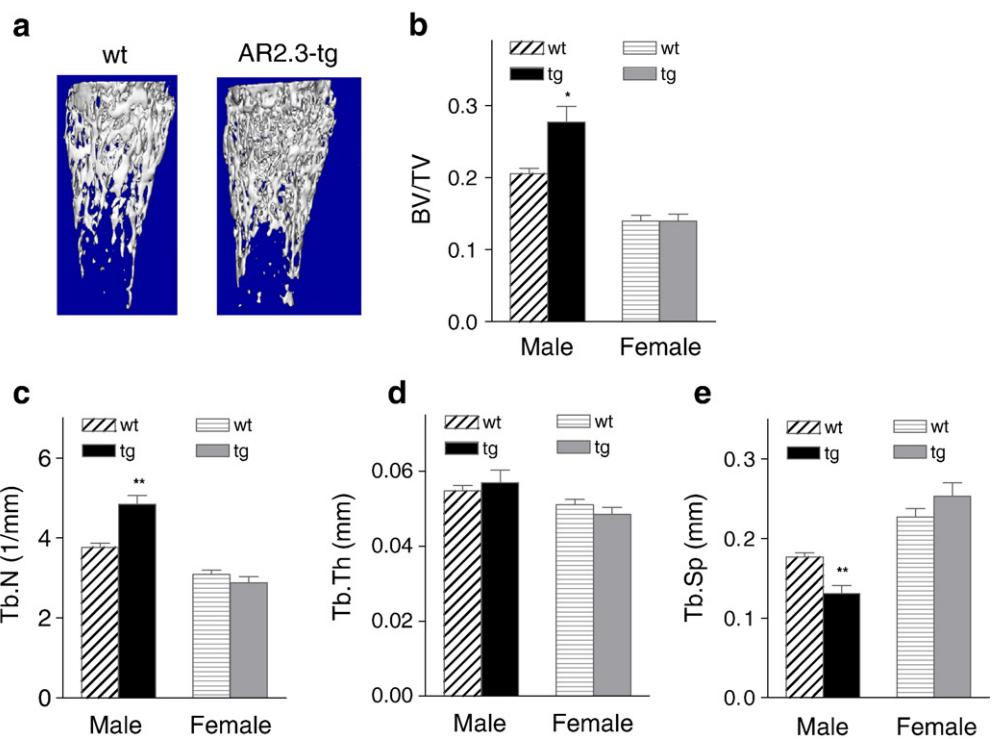
Fig. 5. Trabecular morphology and micro-architecture in AR2.3-transgenic mice. Computer-aided analysis of µCT images was used to derive measures of trabecular bone micro-architecture in the metaphysis of 2-month-old male and female wild-type (wt) or AR2.3-transgenic (tg) mice. Measurements included trabecular bone volume as a percent of tissue volume (BV/TV); trabecular number, spacing, and thickness (Tb.N, Tb.Sp, Tb.Th). a. Reconstructed images were evaluated for trabecular morphology in the distal metaphysis. b. BV/TV. c. Tb.N. d. Tb.Th. e. Tb.Sp. Values are expressed as mean±SEM, n=10–21 males; 13–19 females. Differences between genotypes were determined by Student's unpaired t-test with Welch's correction. *P<0.05; **P<0.01 (*vs.* gender-appropriate wt controls).
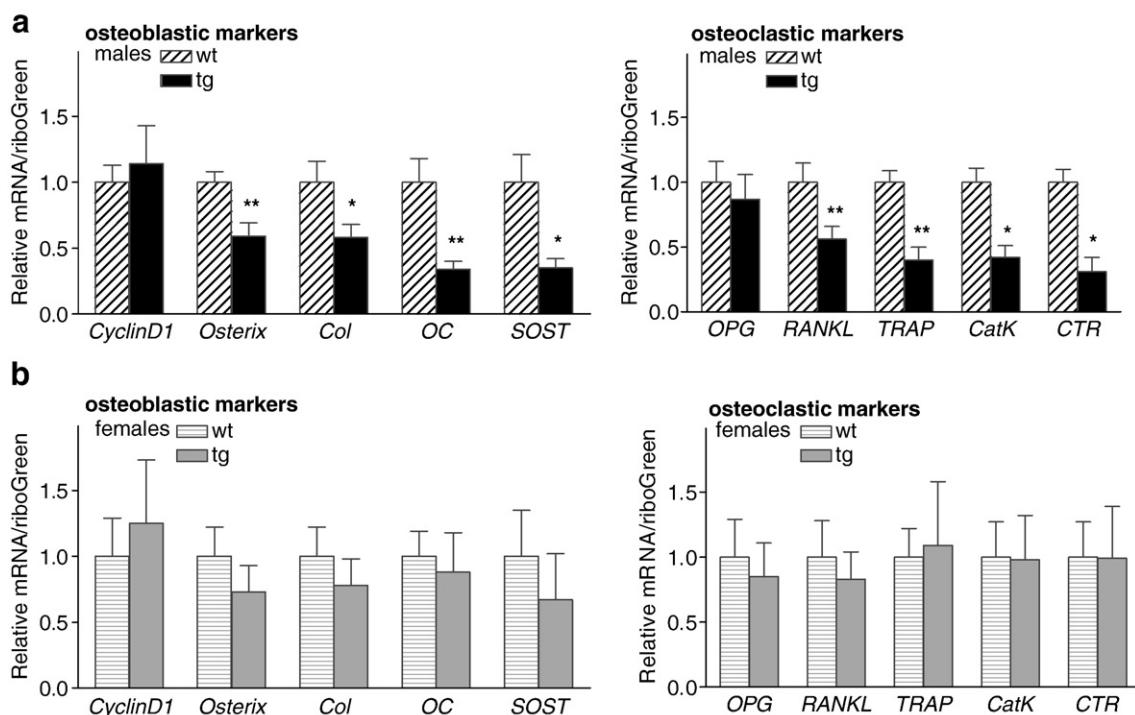
Fig. 6. Cortical bone gene expression in AR2.3-transgenic mice. Analysis of steady-state mRNA expression for genes involved in bone formation or bone resorption was determined by real-time qRT-PCR analysis using tibial RNA isolated from male and female wild-type (wt) or AR2.3-transgenic mice (tg). Osteoblastic marker genes involved in bone formation and matrix production examined included cyclin D1, osterix, type I α_1 collagen (Col), osteocalcin (OC) and sclerostin (SOST). Osteoclastic marker genes involved bone resorption and osteoclastic activity were osteoprotegerin (OPG), RANK ligand (RANKL), tartrate-resistant acid phosphatase (TRAP), calcitonin receptor (CTR) and cathepsin K (CatK). a. Examination of osteoblastic and osteoclastic marker gene expression in male mice. b. Analysis in females. n=3–8 males; 4–7 females. Values are expressed as mean±SEM. *P<0.05, **P<0.01.

of a bone phenotype in females, there was little difference in expression in female transgenics compared to control mice for any of the osteoblastic or osteoclastic marker genes analyzed (Fig. 6b).

Discussion

The specific role of androgen signaling though transactivation of the AR in maintenance of skeletal homoeostasis remains controversial. To determine the specific physiologic relevance of androgen action in the mature osteoblast/osteocyte population in bone, mice with targeted AR overexpression in mature osteoblasts were developed. Characterization of the consequences of bone-targeted overexpression revealed a skeletal phenotype in male transgenic mice vs. littermate controls, with little difference between the females. Collectively, the phenotype observed in male transgenic mice is likely dependent on the higher serum levels of testosterone (~10-fold) in males vs. females. In this study we have found that AR overexpression in the mature osteoblast population in vivo results in a low turnover state, with increased trabecular bone volume but a significant reduction in cortical bone area due to inhibition of bone formation at the endocortical surface and a lack of marrow infilling. Combined, our results indicate that AR overexpression in mature osteoblasts/osteocytes inhibits endocortical bone formation, and results in changes that are detrimental to matrix quality, biomechanical competence and whole bone strength.

Among the most striking biomechanical characteristics of the bone from male AR2.3-transgenic mice was its markedly impaired fracture resistance. Brittleness is a measure of the amount of deformation a structure undergoes prior to failure, and long bones were significantly more brittle and consequently showed large decreases in work-to-failure. The observed inhibition in bone quality appears to be principally determined by changes in the organic matrix of bone, through the ~50% reduction in MAR at the endocortical surface (which reflects osteoblast vigor or work). Such a robust reduction would lead to detrimental changes in the composition of the material properties of the organic matrix, and thus a worsening in post-yield deflection and work-to-failure. Brittleness, and its opposite, ductility, are functional attributes that in bone derive principally from matrix composition and collagen organization rather than bone geometry and mass, which are the major determinants of bone stiffness and strength. The increased brittleness (i.e., decreased post-yield deflection or ductility) of cortical bone is not likely due to over-mineralization, since TMD was reduced in transgenic males, not increased. Thus, reduced ductility observed in these AR-transgenic mouse bones points to a defect in bone matrix quality. This in turn suggests a defect in osteoblast production of a functionally appropriate bone matrix in the presence of enhanced androgen signaling in mature osteoblasts/osteocytes. Indeed, our molecular analysis of expression differences from the AR-transgenic mice show dramatically reduced collagen and osteocalcin production, consistent with the impaired matrix quality in these mice.

It is instructive to compare the skeletal phenotypes that develop in the two distinct lines that we have generated, the AR2.3-transgenic mice described in these studies and the previously characterized AR3.6-transgenic model [67]. In broad terms, the skeletal phenotype characterized in AR2.3-transgenic mice mirrors that described previously for AR3.6-transgenic males, indicating the reproducibility of the phenotypic consequences of bone-targeted androgen signaling. In common between the two models, we have shown increased trabecular bone volume, reduced formation at endocortical surfaces, reduced bone turnover and compromised biomechanical strength in male transgenic mice. With the exception of enhanced periosteal activity in AR3.6-transgenic males, neither model exhibits anabolic bone formation responses in the cortical bone compartment and instead both show inhibition of bone formation at the endocortical surface. Both also demonstrate significantly compromised biomechanical properties. By comparing and contrasting the two AR-transgenic models, we propose that the commonalities in the bone phenotype

between AR2.3-transgenic and AR3.6-transgenic mice arise from AR overexpression in mature osteoblasts and osteocytes, since both promoters are active in these cells. Thus, bone phenotypic changes likely to be mediated at least in part by enhanced androgen signaling in mature osteoblasts/osteocytes include reduced bone turnover, increased trabecular bone volume, reduced endocortical bone formation with decreased osteoblast vigor at endocortical surfaces, and compromised biomechanical strength with increased bone fragility.

The most striking contrast between the two AR-transgenic models is observed at periosteal surfaces in AR3.6-transgenic males, which show increased cortical bone formation in the periosteum and dramatic intramembranous calvarial thickening. This finding was expected, given col3.6 transgene targeting to the periosteum and, conversely, the lack of expression at the same compartment with col2.3 transgene expression [25,26]. The specificity of the periosteal anabolic effect on bone formation in AR3.6-transgenic males is consistent with previous reports documenting the importance of androgen signaling in periosteal expansion [60]. During development, girls and boys build mechanically functional structures (i.e., the size, shape and quality of the bone appears to be well-matched for the size of the individual), but by different means [51]. During/after puberty, an increase in estrogen in girls leads to reduced periosteal expansion and then a reversal on the endosteum, from expansion to infilling. In boys, testosterone levels increase, which in contrast to girls is associated with further growth of the periosteum but also continued expansion of the endocortical cavity. Consequently, the outer diameter of girls' bones tends to be smaller than that of boys' bones and greater cross-sectional area is observed in males [13], yet cortical thickness is similar between males and females (but see[41,62]) because of adaptive infilling in females. Thus, we propose that androgen inhibition of medullary bone formation at the endocortical surface in males may subserve an important physiological adaptive function, being the key for appropriate spatial distribution and maintenance of the total amount/weight of bone in the cortical envelope. A reasonable hypothesis is that androgens strongly promote the addition of cortical width through periosteal growth, but balance that growth with inhibition in
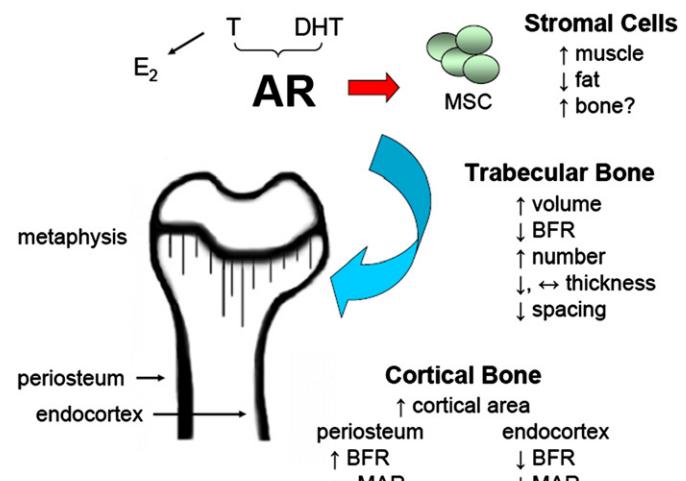
Fig. 7. Model for androgen action in the skeleton mediated by AR transactivation. Androgen activation of AR influences a variety of target organs and skeletal sites, including marrow stromal cells, and trabecular, cortical and intramembranous bone compartments. Arrows indicate the changes associated with androgen action. In trabecular bone, androgen action preserves or increases trabecular number, has little effect on trabecular thickness, and thus reduces trabecular spacing. In cortical bone, AR activation results in reduced bone formation at the endocortical surface but stimulation of bone formation at the periosteal surface. Summary based on results presented here and references cited in the text. In the transgenic model, AR activation in mature bone cells in vivo results in a low turnover phenotype, with inhibition of bone formation and inhibition of gene expression in both osteoblasts and osteoclasts. In the absence of compensatory changes at the periosteal surface, these changes are detrimental to overall matrix quality, biomechanics and whole bone strength.

the marrow cavity so that the skeleton does not become too heavy (see [9]). Based on our characterization of AR-transgenic mouse models and other published reports, we propose a model for the consequences of androgen signaling where the effects of AR activation are distinct in different skeletal compartments (Fig. 7). In trabecular bone, androgens reduce bone formation [58] and suppress resorption to increase trabecular volume through an increase in trabecular number. In cortical bone, androgens inhibit osteogenesis at endocortical surfaces but increase bone formation at periosteal sites [67], to maintain cortical thickness yet displace bone further away from the neutral axis in males. Androgens also positively influence bone growth at intramembranous sites [16,67]. In addition to actions directly in bone, androgen administration also increases muscle mass, partially mediated by effects on mesenchymal stem cell lineage commitment [52]. This increase in muscle mass may indirectly influence bone density through biomechanical linkage. Additional studies will be needed to more fully test these hypotheses.

Male AR-transgenic mice also demonstrate a phenotype consistent with reduced osteoclast resorptive activity. In cortical bone, both TRAP and RANKL gene expression is inhibited, and serum levels of both TRAPC5b and CTx are also significantly reduced in transgenic males. In addition, the observed increase in trabecular bone volume with a decrease in trabecular separation is a hallmark of antiresorptive activity. However, future studies employing dynamic histomorphometric analysis will be needed to verify these results. Potential modulation of osteoclast action by DHT is incompletely characterized, although there are reports of AR expression in the osteoclast [57]. Androgen may be a less significant determinant of bone resorption *in vivo* than estrogen [14], but this remains controversial [34]. The bone phenotype that develops in a global AR null male mouse model, a high-turnover osteopenia with reduced trabecular bone volume and a stimulatory effect on osteoclast activity [28,29,69], also supports the importance of androgen signaling through the AR to influence resorption, and is generally opposite to the phenotype we observe with skeletally targeted AR overexpression. Interestingly, the global AR null model also develops late onset obesity [15]. Finally, recent publications document that androgen can directly reduce bone resorption of isolated osteoclasts [45], inhibits osteoclast formation stimulated by PTH [10], and may play a direct role regulating aspects of osteoclast activity in conditional AR null mice [40]. Our results suggest that at least some component of inhibition of osteoclastic resorptive activity as a consequence of androgen administration is mediated indirectly through effects on mature osteoblasts and osteocytes.

Some of the negative consequences of AR overexpression in mature osteoblasts we have observed *in vivo* may reflect previously documented *in vitro* analyses. For example, there are reports, some in clonal osteoblastic cell lines, of effects of gonadal androgen treatment on differentiation, matrix production and mineral accumulation mediated by AR signaling [3,27,53]. These findings are variable however, with other reports of no effect or even inhibition of osteoblast markers [6,18,21], consistent with our gene expression analysis in AR-transgenic mice. In addition, the effect of androgens on osteoblast proliferation is controversial. We have previously demonstrated that either stimulation or inhibition of osteoblast viability by androgen can be observed, and these effects are dependent on the length of treatment. Transient administration of nonaromatizable DHT can enhance transcription factor activation and osteoblast proliferation, while chronic treatment inhibits both mitogenic signaling and MAP kinase activity [66]. Chronic DHT treatment *in vitro* can also enhance osteoblast apoptosis [65]. Combined, these *in vitro* reports are consistent with the detrimental changes in matrix quality and osteoblast vigor we observe in the AR-transgenic model *in vivo*.

In summary, complex skeletal analysis using morphological characterization by μCT, dynamic and static histomorphometric analysis, DXA, biomechanical testing and gene expression studies all indicate that AR overexpression in mature osteoblasts inhibits osteogenesis at endocortical surfaces and produces a low turnover state. Importantly, these changes are detrimental to overall matrix quality, biomechanical competence, bone fragility and whole bone strength. It is possible that the observed inhibition of endocortical osteogenesis and lack of anabolic response, as a consequence of enhanced androgen signaling in mature bone cells, underscores an important physiological function for androgen in the skeleton: to maintain an appropriate spatial distribution of bone in the cortical envelope. Androgens are able to maintain trabecular bone mass and are effective in the treatment of bone loss associated with hypogonadism. Nevertheless, the strong inhibition of bone formation at the endocortical surface and increased bone fragility observed here highlight compartment-specific responses that might underlie the limited therapeutic benefits observed with androgen therapy. Because of the detrimental consequences of bone-targeted androgen signaling on bone fragility and whole bone strength, these results raise concerns regarding anabolic steroid abuse or high-dose androgen therapy during growth and in healthy eugonadal adults.

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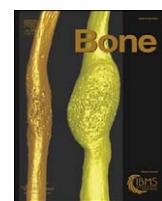
Signaling pathways implicated in androgen regulation of endocortical bone

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ABSTRACT

Periosteal expansion is a recognized response to androgen exposure during bone development and in profoundly hypogonadal adults. However, androgen also suppresses endocortical bone formation, indicating that its effects on bone are dichotomous and envelope-specific. In fact, enhanced androgen signaling has been shown to have dramatic detrimental effects on whole bone biomechanical properties in two different transgenic models with skeletally targeted androgen receptor (AR) overexpression. As the mechanisms underlying this response are uncharacterized, we compared patterns of gene expression in periosteum-free cortical bone samples derived from AR-overexpressing transgenic male mice and their wild-type counterparts. We then assessed direct androgen effects in both wild-type and AR-overexpressing osteoblasts in primary culture. Among major signaling pathways associated with bone formation, focused quantitative RT-PCR (qPCR) array-based analysis of endocortical bone gene expression from wild-type vs. transgenic males identified the transforming growth factor-beta (TGF-β) superfamily and bone morphogenetic protein (BMP) signaling as significantly altered by androgen *in vivo*. Bioinformatic analyses indicated proliferation, osteoblast differentiation and mineralization as major biological processes affected. Consistent with the *in vivo* array data and bioinformatic analyses, inhibition of differentiation observed with androgen exposure was reduced by exogenous BMP2 treatment of AR-overexpressing cultures to stimulate BMP signaling, confirming array pathway analysis. In addition, nonaromatizable dihydrotestosterone (DHT) inhibited osteoblast proliferation, differentiation and several indices of mineralization, including mineral accumulation and mineralized nodule formation in primary cultures from both wild-type and AR-transgenic mice. These findings identify a molecular mechanism based on altered BMP signaling that contributes to androgen inhibition of osteoblast differentiation and mineralization. Such detrimental effects of androgen on osteoblast function may underlie the generally disappointing results of androgen therapy.

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Introduction

Androgen has historically been considered a bone anabolic agent [1]. Androgen deficiency typically results in osteopenia, while repletion of androgen-deficient males or androgen supplementation of osteoporotic females has been shown to reduce bone loss [2]. These observations have been reproduced in animal models of androgen deprivation and androgen receptor dysfunction (for review, see [3]). Yet, the specific effects of androgen on the skeleton *in vivo* remain controversial. For example, androgen supplementation has little positive effects on bone formation in non-hypogonadal adults [4] or in normal intact adult animal models, and results from clinical trials attempting to build bone mass have been relatively disappointing [5]. In addition, two models where the androgen receptor has been selectively overexpressed in skeletal cells reveal that androgen exerts a complex combination of positive and negative effects on bone that are compartment-specific. As demonstrated in skeletally targeted transgenic male mice in which AR overexpression is driven by either 2.3 or 3.6 kb col1α1 promoter constructs, hormone-dependent reductions in overall bone turnover indices at the endocortical envelope as well as impaired whole bone biomechanical properties including reduced strength, stiffness, post-yield deflection and work-to-failure are observed at the femoral midshaft [6,7]. Only the AR3.6-transgenic male mice, which unlike AR2.3-transgenic mice over-express AR in the periosteum, show significantly increased periosteal formation compared to wild-type littermate controls [6]. Combined, these studies clearly demonstrate androgen effects are envelope-specific and that androgen signaling can act to suppress bone formation *in vivo*.

The molecular and cellular mechanisms whereby androgen action leads to these complex skeletal phenotypes remain unclear and relatively unexplored. Although a variety of approaches have been employed to characterize androgen responses *in vivo*, tissue-selective modulation of AR expression may be the most mechanistic approach

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to determine direct androgen action in the skeleton. Characterization of the effects of both overexpression and targeted deletion of AR has been described, and generally opposite phenotypes are observed most notably in trabecular bone. Knockdown of genomic AR signaling in mature osteoblasts results in cancellous osteopenia, with increased bone resorption, a reduction in trabecular bone volume and a decrease in trabecular number, indicating the importance of AR signaling to maintain trabecular bone [8,9]. Consistent with and opposite to these findings, targeted AR overexpression also results in a bone phenotype in the two distinct transgenic lines noted above. The AR3.6-transgenic mice show AR overexpression in osteoblast stromal precursors and throughout the osteoblast lineage [6]. A major advantage of this model is overexpression of AR in the periosteal compartment, a known target for androgen anabolic action in the skeleton. By comparison, the AR2.3-transgenic mice have overexpression of AR that is restricted to mature osteoblasts and osteocytes [7]. Since osteocytes are the most abundant cell type in bone [10] and also have the highest concentration of AR [11], these cells are also likely an important target cell for androgen action and may represent a mediator for skeletal responses to testosterone therapy in vivo. In general, bone-targeted AR overexpression in vivo in both models results in a low turnover state with a significant reduction in cortical bone area due to inhibition of bone formation at the endocortical surface and a lack of marrow infilling. With the exception of periosteal bone formation in AR3.6 transgenic mice, there is no anabolic bone response in either AR overexpression model. Opposite to AR null models, enhanced AR signaling increased trabecular bone volume via an increase in trabecular number but not width, with reduced osteoclast number and/or activity. Finally, results from both models indicate that enhanced androgen signaling in bone results in overall changes that combined are quite detrimental to biomechanical competence and whole bone strength, at least partly due to reductions in osteoblast vigor and organic matrix quality as well as changes in geometry. Based on these findings, we have suggested that androgen inhibition of medullary bone formation at the endocortical surface in males may subserve an important physiological adaptive function, being key for balancing the total amount/weight of bone in the cortical envelope while maintaining appropriate spatial distribution [7]. In vivo, few effects of androgen on the endocortical envelope have been described in other models, and the molecular or cellular basis for this dramatic inhibition of endocortical bone formation has yet to be established.

Evidence does suggest that androgens act directly on the osteoblast to influence gene expression and function. However, likely as a consequence of the complexity of osteoblast differentiation and the various models employed, reports describing how androgen influences bone cell function are inconsistent and the literature remains controversial. Thus, in vitro investigations of androgen effects that generally employ immortalized or passaged osteoblastic cell models have yielded conflicting results, with either positive or negative effects on proliferation [12–17], gene expression and differentiation [15,17–20], and apoptosis [21,22]. Androgen treatment has been reported to enhance production of mineralized matrices in vitro [18,20,23,24]. Thus, little consensus has been developed with regard to the effects of androgens on osteoblast proliferation and differentiation in vitro. In previous studies, we investigated the effects of enhanced androgen signaling in immortalized osteoblastic cell lines stably transfected with an AR expression construct, and demonstrated hormone-dependent reductions in growth, as well as changes in ERK$_{1,2}$ and the MAPK cascade [16]. In addition, we found that while estradiol reduced, chronic androgen treatment enhanced apoptosis in both normal wild-type and overexpressing cells, which was associated with an increase in the Bax/Bcl-2 ratio [21]. Increased apoptosis was also observed in vivo in AR-transgenic calvaria, in a hormone-dependent fashion, in both young and adult mice at 2 months and 6 months of age respectively [21].

In the present study, we sought to characterize mechanisms underlying androgen action in the skeleton. To identify the basis or consequences of androgen-mediated suppression of bone formation in vivo, alterations in molecular signatures in endocortical bone were characterized by qPCR array analysis with a focus on pathways previously associated with bone formation. Differences between male AR-overexpressing mice and their wild-type counterparts in the expression patterns of osteoblast-associated genes was characterized employing endocortical bone samples from which periosteum, trabecular and marrow elements had been removed. For biological confirmation of the signaling pathways identified in vivo, and to characterize potential cell autonomous effects of androgen treatment, the functional consequences of androgen signaling in osteoblastic cells was evaluated in normal primary cultures isolated from wild-type and from AR-transgenic animals.

Materials and methods

Real-time quantitative reverse transcription-polymerase chain reaction (qPCR) array and bioinformatic analyses

Transgenic mice offer the advantage of skeletal targeting and do not show alterations in circulating sex steroid concentrations that could indirectly alter bone metabolism through effects on other organ systems. Although both transgenic lines showed reduced bone formation at the endocortical surface [6,7], the AR3.6-transgenic line was used as a source to identify androgen-regulated transcripts in endocortical bone because the phenotype is more robust. All experiments involving animals were approved by the Portland VA Medical Center IACUC. Males were employed because the female cohort does not demonstrate a significant bone phenotype, likely a consequence of low endogenous androgen concentrations in females relative to males. Mice representing both independently derived AR3.6-transgenic families (104 and 106) at 3–4 months of age were employed for these analyses. To isolate endocortical bone RNA, femora and tibiae were dissected from five to six mice of each genotype and the periosteum was removed by five sequential enzymatic digestions at 37°C each for 30 min using a mixture of collagenase type II and trypsin (0.1% and 0.125%) in Hanks' Balanced Salt Solution (HBSS) (Invitrogen Corp., Carlsbad, CA). After digestion, the metaphyses were removed to eliminate trabecular bone and the marrow cavity was flushed with sterile HBSS. The remaining endocortical bone was ground under liquid nitrogen and RNA was extracted with the single-step acid guanidinium isothiocyanate–phenol–chloroform extraction method using RNA Stat-60 (Tel-Test, Inc., Friendswood, TX). Contaminating DNA was removed from total RNA by DNase I treatment and Zymo-spin column purification following the manufacturer's recommendations (Zymo Research, Orange, CA). RNA integrity was confirmed on a 1% agarose gel stained with SYBR Gold Nucleic Acid Gel Stain (Molecular Probes, Invitrogen Corp.). A two-step RT-PCR reaction employed 500 ng total RNA per 20 μl reaction with a mix of random and oligo-dT primers, using qScript cDNA Supermix (Quanta Biosciences, Gaithersburg MD). Gene expression differences between wild-type and AR-overexpressing tissues were assessed using commercially available, pre-validated pathway-specific gene expression mouse qPCR arrays (StellARray, Bar Harbor Biotechnology, Inc., Trenton, ME) for each AR3.6-transgenic family. The analysis was replicated to determine statistical differences and represents collapsed data across both AR3.6-transgenic families for comparison between genotypes. Four 96-well arrays chosen to create a 384-well plate with amplicons targeting bone homeostasis for transcripts involved in (i) osteoporosis, (ii) targets of Wnt/β-catenin signaling containing Tcf binding sites, (iii) TGF-β signaling and (iv) NFκB signaling. A list of the 384 genes is available online at http://array.lonza.com. Expression changes were assayed simultaneously by qPCR. The qPCR reactions and subsequent quantitative

analysis were performed by Bar Harbor Biotechnology, Inc. Expression difference was determined after normalization without predefined normalizers using a Global Pattern Recognition (GPR) algorithm [25] that determines invariant genes for normalization in every experiment. Significance was determined in an iterative process using a two-tailed heteroscedastic t-test, with a $p < 0.05$ considered significant using a weighted geometric mean. The cutoff for expression was at 37.5 cycles. This approach uses a statistical gene ranking system without fold change bias. Overrepresented functional groups were identified using WebGestalt Gene Set Analysis Toolkit software at http://bioinfo.vanderbilt.edu/webgestalt [26], with the 384 genes on the custom array used as the background gene list for GO pathway analysis. Regulated biological networks were identified using pathway analysis (IPA v7.0; Ingenuity Systems, http://www.ingenuity.com), based on our specific user dataset as the reference set for comparison. As a third independent bioinformatic analysis, Pathway Architect software (GeneSpring GX10, Agilent Technologies, http://www.chem.agilent.com) was used to identify associations among the differentially expressed genes and biological processes for follow-up confirmation.

Primary calvarial cultures

Normal primary osteoblastic cultures were derived from calvaria harvested from 3 to 6 day old wild-type, AR3.6-transgenic and AR2.3-transgenic mice by sequential collagenase-P digestions. Calvariae were pooled after genotyping. The parietal and occipital bones were dissected free of the suture lines and subjected to four sequential 15-min digestions in a mixture containing 0.05% trypsin and 0.1% collagenase-P at 37°C. Cell fractions 2–4 were pooled and plated at 8000 cells/cm^2 in MEM media supplemented with 10% FBS. The medium was changed at 24 h with 10% charcoal-stripped FBS and treated continuously with vehicle or DHT at 10^{-8} M for indicated time points. Beginning at confluence (day 7), cultures were switched to differentiation medium in phenol-red free BGJb (Fitton-Jackson modification) supplemented with 10% charcoal-stripped FBS containing 50 μg/ml ascorbic acid. From day 14 on, 5 mM β-glycerophosphate was added to the differentiation media. Steroids were dissolved as stocks in ethanol and used at concentrations 10^{-8} M. The final ethanol concentration in the media was no higher than 0.1%. BMP2 (R&D Systems, Minneapolis, MN) was used at 100 ng/ml in 4 mM HCl/0.1% bovine serum albumin. All other media, buffers, supplements and reagents for cell culture were obtained from GIBCO BRL-Life Technologies (Grand Island, NY) and Sigma Chemical Co. (St Louis, MO).

Confirmation of expression differences in primary cultures by qPCR analysis

Since screening of all differentially expressed genes was not feasible, we selected five genes of interest for further confirmatory analysis in osteoblastic cells. Genes were selected to represent both up- and down-regulation, and span a range of statistical significance, fold expression difference, or biological interest and potential involvement in BMP signaling. RNA was harvested from mature primary cell cultures derived from AR3.6-transgenic mice treated continuously with 10^{-8} M DHT. The qPCR reactions were carried out in 25 μl with 20 ng of total RNA in a reaction mix containing 1× QuantiTect SYBR Green RT-PCR Master Mix (Qiagen, Valencia, CA) and 0.5 μM each primer. Real time qPCR was performed with the iCycler IQ Real Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA) using a one-step QuantiTect SYBR Green RT-PCR kit (Qiagen) on DNase-treated total RNA. Relative expression of the PCR product was determined using the comparative ΔΔCt method, after normalizing expression to total RNA measured with RiboGreen (Molecular Probes) as previously described [27]. Data are presented as fold change

relative to control samples. Primers for calvaria RNA analysis were purchased pre-designed from Qiagen. Following PCR, reaction products were melted over the temperature range 55°C to 95°C in 0.5°C increments, 10 s per increment to ensure only the expected PCR product was amplified per reaction. The efficiency of amplification was determined for each primer set from serial dilutions, and did not vary significantly from 2.

Analysis of AR protein levels by Western blot

Whole cell lysates were collected from primary cultures during in vitro differentiation at 7, 14, 21 or 28 days post-plating. AR abundance was determined by immunoblotting with rabbit polyclonal AR antibody (ARN-20; Santa Cruz Biotechnology, Santa Cruz, CA) as previously described [21]. As a loading control, a mouse monoclonal α-tubulin antibody (T9026; Sigma) was used at 1:1000. Bound antibodies were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) on Kodak X-AR5 autoradiographic film. Quantitative analysis of the proteins was performed by volume densitometry using OptiQuant software (Perkin-Elmer, Waltham, MA) after scanning of the film in the linear range. Data are presented as the protein to α-tubulin ratio to correct for variations in protein loading, and then normalized to values from wild-type at day 7 to determine fold change for comparison between the different time points.

Confirmation of pathway analysis identifying inhibition of BMP signaling

The ability of exogenous BMP2 to override the inhibitory effects of androgen treatment on osteoblast differentiation was evaluated in primary cultures, assessed by alkaline phosphatase (ALP) activity. Cells were treated continuously with vehicle, nonaromatizable dihydrotestosterone (DHT) at 10^{-8} M, 100 ng/ml BMP2 or the combination of DHT and BMP2 at those doses.

Cell growth and differentiation analysis

Cell number was assessed in the absence of hormone treatment. Cells were harvested at 24 h intervals after trypsinization and counted using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL). The effects of DHT on osteoblast proliferation were determined using the BrdU (5-bromo-2′-deoxyuridine) Cell Proliferation Assay kit (Calbiochem, La Jolla, CA). Primary cultures were grown in 96-well dishes for 5 days with vehicle or 10^{-8} M DHT. BrdU was added to test wells during the final 20 h, and then measured as arbitrary units of optical density (OD) at 450 nm with a microplate reader (Model 550; Bio-Rad). Modulation of differentiation was assessed by staining for ALP and alizarin red-S (AR-S) accumulation. For ALP histochemical staining, cells were rinsed with phosphate buffered saline, fixed with citrate-acetone formaldehyde fixative, rinsed and stained for enzyme activity with 0.25% AS-BI phosphate alkaline solution (Sigma). AR-S staining is described below.

ALP activity and mineral accumulation

Primary cultures were treated with vehicle or 10^{-8} M DHT continuously. ALP activity was determined at day 14 after washing twice with ice-cold phosphate-buffered saline, then harvesting in 1 ml 50 mM Tris–HCl (pH 7.6), sonicating twice on ice and then centrifuging at 4°C for 15 min at $1000 \times g$. The supernatants were stored at −20°C until analysis for ALP activity using p-nitrophenyl-phosphate as substrate. Absorbance was read at 405 nm using a microplate reader (Model 550; Bio-Rad). ALP activity was measured as nmol p-nitrophenol released per min per μg protein. Analysis of mineralization was determined by alizarin red-S (AR-S) staining and accumulation, and by von Kossa staining. Cells were rinsed with HBSS,

fixed for 2 h in 70% ethanol at 4°C, rinsed with 1 mM HEPES and calcium deposition was then visualized after incubation with 4 mM AR-S pH 4.2. AR-S was extracted by destaining with 10 mM HCl in 70% ethanol, and mineral accumulation was quantified on a microplate reader at 520 nm. The cell layer was then solubilized in lysis buffer (10 mM formamide, 50 mM sodium acetate, 1% SDS, pH 6) and protein content was assessed with BCA protein assay reagent (Pierce, Rockford, IL). Results were expressed as nmol alizarin red-S per μg protein. Cultures were also analyzed simultaneously for ALP histo-chemical staining and mineral staining by von Kossa in 1.88 cm^2 wells (4-well Lab-Tek chamber plates) at 21 days after continuous treatment with vehicle or 10^{-8} M DHT. Cells were first subjected to ALP histochemical staining, then plates were rinsed briefly with water and covered with 5% AgNO$_3$ for 1 h under bright light. Bone nodules were visualized by light microscopy and quantified from digital photographs using LAS v3.3.0 software (Leica Microsystems Inc., Bannockburn, IL).

Statistical analysis

Data were analyzed using Prism software v5.02 (GraphPad Software, Inc., San Diego, CA). Our *a priori* hypothesis was that genotype would be differentially responsive to androgen treatment. Thus, significance of difference between treatment groups in the absence of an interaction with genotype was assessed by an unpaired two-tailed *t*-test or one-way ANOVA, followed by post-hoc testing with Neuman–Keuls Multiple Comparison Test when ANOVA demonstrated significant differences. Confirmation of differential gene expression employed a one-tailed *t*-test. All data is expressed as mean ± SD and all experiments were performed two to four times. $P < 0.05$ was considered significant.

Results

Gene profiling analyses to characterize pathways influencing androgen inhibition of cortical bone formation

To gain an understanding of the transcriptional basis for the observed inhibition of osteoblast function as a consequence of androgen signaling and to identify important signal transduction pathways that are physiologically relevant, we used *in vivo* gene profiling analysis. Targets of androgen signaling were identified via characterization of expression differences in long bones harvested from male AR3.6-transgenic or wild-type control mice. Endocortical bone samples were isolated from long bone after enzymatic stripping of the periosteum, removal of the metaphyses to eliminate trabecular bone and flushing of the marrow cavity. Total RNA was then harvested. Gene expression was surveyed using qPCR arrays for identification of regulated transcripts, with a targeted qPCR array containing 384 genes. Because little is known about androgen regulation of gene expression in bone, we constructed the array to contain pathways with established importance in bone development, formation and remodeling with a focused panel of amplicons for genes with well-characterized roles in osteoporosis, TGF-β/BMP signaling, NFκB signaling, and targets of Wnt/β-catenin signaling. Thus, the qPCR array analysis tested the hypothesis that inhibition of endocortical formation by androgen may be mediated by pathways that have been previously associated with bone formation.

Of the 384 array genes examined by qPCR analysis, expression of a total of 78 genes (20%) were significantly different between wild-type and transgenic bones. Significantly up-regulated sequences are listed in Table 1 and down-regulated sequences are listed in Table 2. Notably and as expected, AR expression was elevated in the transgenic bones. The most up-regulated transcripts were Casr, Nov (CCN3), Bmp15, Comp and Gdf5 while the most down-regulated transcripts were

MCHR 1, neurogenin 1, inhibin-β C, interleukin-6 and Traf3. Fig. 1A shows a volcano plot where differentially expressed genes were arranged along dimensions of biological impact (fold change) versus statistical significance (for reliability of change). The horizontal dimension shows fold change between the two groups and the vertical axis represents the *p*-value for a *t*-test of differences between samples. We also confirmed expression differences by qPCR in independent samples derived from primary calvarial osteoblast cultures treated continuously with 10^{-8} M DHT for selected genes. As shown (Fig. 1B), significant differences were observed in DHT treated samples for Sox9, TGF-β2, Runx2, Six3 and collagen and are thus androgen-regulated in primary osteoblasts as well as in intact bone. This result is consistent with the notion that changes in expression derived from bone samples arise in osteoblasts in a cell autonomous process.

We then used three independent computational approaches to identify the biological targets that are impacted by these expression differences: significantly enriched gene ontology (GO) categories were identified using WebGestalt software and biological processes were characterized using Ingenuity Pathway Analysis (IPA) and Pathway Architect software. Of the GO categories identified, the most significantly regulated were negative regulation of BMP signaling ($p = 0.012$) and regulation of mitosis ($p = 0.037$). Thus, the pathway that was most significantly modulated was BMP signaling, which includes genes for chordin, SMAD specific E3 ubiquitin protein ligase 1 (Smurf1), Six3 and sclerostin (SOST). Examination of expression differences highlights the number of sequences for genes involved in TGF-β/BMP/Activin signaling pathway influenced by androgen in bone, summarized in Fig. 2. Notably, many (but not all) of the up-regulated sequences function in an inhibitory fashion to block pathway activation. The cellular function most significantly regulated was mitosis, with genes including baculoviral IAP repeat-containing 5 (Birc5), ataxia telangiectasia mutated homolog (Atm) and neurogenin 1 (Neurog1). To identify associations among the significantly regulated genes and characterize biological processes that were impacted by androgen signaling in mature osteoblasts/osteocytes, we employed additional bioinformatic analyses to identify associations among the regulated genes. IPA analysis was used to characterize associated network functions, and identified "Skeletal and Muscular Disorders, Skeletal and Muscular System Development and Function, Tissue Development" with the highest score. The higher the score, the more interconnected the regulated molecules are within a given network. Finally, a third independent analysis was performed using Pathway Architect software (Supplementary Figure 1). Examination of the results revealed several notable findings, including proliferation and negative regulation of osteoblast differentiation (consistent with cellular function analysis above), regulation of mineralization, inhibition of osteoclastogenesis/activity and regulation of chondrogenesis, all identified as biological themes targeted by androgen in cortical bone. Combined, these results prompted us to examine more closely androgen-associated changes in osteoblast proliferation, differentiation and mineralization.

Phenotypic characterization of calvarial osteoblasts/osteocytes from AR-transgenic mice

As noted above, the array analysis was derived from whole bone samples so that some of the expression changes we have identified may have been a consequence of adaption to altered bone morphology and not direct androgen regulation of the osteoblast/osteocyte. Thus, in order to more fully characterize the cell autonomous effects of direct androgen signaling in osteoblasts, we employed normal primary cultures derived from wild-type and both AR-transgenic lines since males from both transgenic lines show reduced endocortical bone formation [6,7]. Primary osteoblastic

Table 1

Up-regulation of expression in array qPCR analyses in male transgenic mice from cortical bone samples.

Rank	Gene symbol	Gene name (alias)	Change (fold/control)	*p*-value
1	Casr	calcium-sensing receptor	19.28	0.0001
4	Gdf5	growth differentiation factor 5 (BMP14)	5.69	0.001
5	Sox9	SRY (sex determining region Y)-box 9	3.05	0.001
6	Pthr1	parathyroid hormone 1 receptor	2.71	0.002
7	Fzd8	frizzled homolog 8	2.88	0.002
8	Atoh1	atonal homolog 1 (Drosophila)	3.50	0.002
10	Dcn	Decorin	3.19	0.002
11	Nrcam	neuronal cell adhesion molecule	3.40	0.002
14	AR	androgen receptor	5.57	0.005
18	Cyr61	cysteine rich protein 61 (CCN1, IGFBP10)	2.26	0.005
19	Tcf7l2	transcription factor 7-like 2, T-cell specific, HMG-box	2.18	0.006
20	Vdr	vitamin D receptor	2.04	0.006
21	Egr1	early growth response 1	2.23	0.006
23	Tcf4	transcription factor 4	1.91	0.007
24	Inha	inhibin, alpha	3.04	0.008
29	Mmp2	matrix metallopeptidase 2	2.24	0.010
30	Tnfrsf11b	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	1.94	0.011
32	Col1a2	collagen, type I, alpha 2	2.57	0.014
33	Nov	nephroblastoma overexpressed gene (CCN3; IGFBP9)	14.08	0.014
34	Col1a2	collagen, type I, alpha 2	2.64	0.014
35	Bmp15	bone morphogenetic protein 15 (GDF-9B)	10.59	0.015
36	Chrd	Chordin	2.15	0.015
37	Tgfb2	transforming growth factor, β2	1.72	0.016
38	Twist1	twist 1	1.78	0.016
39	Col1a1	collagen, type I, alpha 1	2.82	0.016
44	IGF2	insulin-like growth factor 2	1.88	0.018
45	Ppp1r13l	protein phosphatase 1, regulatory (inhibitor) subunit 13 like	1.90	0.019
46	Serpine1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	1.76	0.019
48	Klf11	Kruppel-like factor 11 (TGF-β inducible early growth response 3)	1.71	0.020
49	Lrp5	low density lipoprotein receptor-related protein 5	1.68	0.021
51	Tgfb2	transforming growth factor-β2	1.59	0.021
52	Fos	FBJ osteosarcoma oncogene	1.69	0.022
53	Smurf1	SMAD specific E3 ubiquitin protein ligase 1	1.59	0.022
54	Bmp8a	bone morphogenetic protein 8a	1.94	0.024
55	Jun	Jun oncogene	1.67	0.024
58	Col1a1	collagen, type I, alpha 1	2.56	0.025
59	Axin2	axin2	1.86	0.025
61	Thbs2	thrombospondin 2	2.11	0.026
62	Nppb	natriuretic peptide precursor B	2.33	0.027
63	Jun	Jun oncogene	1.68	0.027
64	Zfyve9	zinc finger, FYVE domain containing 9 (SARA)	1.58	0.030
67	Runx2	runt-related transcription factor 2 (CBFA1)	1.57	0.032
68	Fos	FBJ osteosarcoma oncogene	1.60	0.033
69	Runx2	runt-related transcription factor 2 (CBFA1)	1.55	0.034
70	Comp	cartilage oligomeric matrix protein	6.45	0.036
72	Runx2	runt-related transcription factor 2 (CBFA1)	1.50	0.038
77	Bmp3	bone morphogenetic protein 3 (osteogenin)	1.59	0.043
79	Smad6	MAD homolog 6	1.49	0.043
82	Sost	Sclerostin	1.51	0.045
83	Cyp17a1	cytochrome P450, family 17, subfamily A, polypeptide 1 (steroid 17-alpha-hydroxylase/17,20 lyase)	1.46	0.046
85	Fhl2	four and a half LIM domains 2	1.63	0.047

Results show genes with significant expression changes when comparing samples after global normalization. Genes are ranked in order of significant changes and listed where the expression change (fold/control) between transgenic and wild-type control was significant at *p* < 0.05, calculated with respect to normalizers based on the GPR algorithm. Analysis was from two samples in replicate representing both independently derived AR3.6-transgenic families.

cells were isolated after genotyping from fetal calvaria and cultures were grown for varying periods up to 28 days, so that cells progressed through proliferation, matrix maturation and finally mineralization stages, to assess androgen actions during *in vitro* differentiation. We first assessed the homogeneity of the culture for presence of osteoblastic cells by analysis of the osteoblastic marker ALP activity, using a histochemical stain. ALP was highly expressed in primary cultures at day 14, with staining intensity in approximately 90% of the cells in culture (data not shown), confirming the specificity of the isolation procedure. We then evaluated the level of AR abundance in primary calvarial osteoblast cultures derived from wild-type or AR2.3-transgenic mice by Western analysis as shown in Figs. 3A and B, as the activity of the AR3.6-transgene has previously been described [21]. AR levels increase during differen-

tiation with the highest levels in the most mature osteoblast/osteocyte cultures, as we have shown previously [11]. The influence of the promoter on total AR levels is seen in more mature cultures after proliferation has ceased, consistent with col2.3 promoter activity [28]. To determine whether transgene expression alone (i.e., without hormone addition for receptor transactivation) influenced proliferation, we then assessed growth characteristics of both wild-type and AR-transgenic cultures in the absence of androgen treatment. Cells were harvested at 24 h intervals and counted. As shown in Fig. 3C, osteoblastic cells from both transgenic and wild-type mice display similar growth kinetics. In addition, we determined differentiation and mineralization capacity of untreated wild-type and AR-transgenic osteoblasts in time-course analysis (Fig. 3D). Cultures were stained for ALP expression by histochemical

Table 2
Down-regulation of expression in array qPCR analyses in male transgenic mice.

Rank	Gene symbol	Gene name	Change (fold/control)	p-value
2	Inhbc	Inhibin-β C	−10.27	1.50E−04
3	Mchr1	melanin-concentrating hormone receptor 1	−21.24	3.30E−04
9	Tiam1	T-cell lymphoma invasion and metastasis 1 (a guanine nucleotide exchange factor that activates Rac)	−2.66	0.002
12	Neurog1	Neurogenin 1	−13.84	0.004
13	Birc5	Baculoviral IAP repeat-containing 5 (member of the inhibitors of apoptosis proteins)	−2.19	0.005
15	Gdf3	Growth differentiation factor 3 (TGF-β superfamily)	−2.26	0.005
16	Itgb2	Integrin, beta 2	−2.31	0.005
17	Il6	Interleukin 6	−3.31	0.005
22	Atm	Ataxia telangiectasia mutated homolog	−1.90	0.007
25	Vwf	Von Willebrand factor homolog (endothelial cell marker)	−2.01	0.009
26	Abcb1b	ATP-binding cassette, sub-family B (MDR/TAP), member 1B (multidrug resistance phosphoglycoprotein)	−2.13	0.009
27	Six3	Sine oculis-related homeobox 3 homolog (inhibition of BMP4)	−1.98	0.010
28	Tradd	TNFRSF1A-associated via death domain	−1.89	0.010
31	Casp1	Caspase 1	−1.86	0.013
40	Tnfrsf11a	Tumor necrosis factor receptor superfamily, member 11a (RANK)	−1.76	0.017
41	Pomc1	Pro-opiomelanocortin-alpha	−1.70	0.017
42	Il1a	interleukin 1 alpha	−1.72	0.018
43	Nkrf	NFκB repressing factor	−2.08	0.018
47	Alox15	arachidonate 15-lipoxygenase	−1.67	0.020
50	Nod1	nucleotide-binding oligomerization domain containing 1	−1.75	0.021
56	Sfrp4	secreted frizzled-related protein 4	−1.82	0.024
57	Eif2ak2	eukaryotic translation initiation factor 2-alpha kinase 2	−1.59	0.025
60	Hdc	histidine decarboxylase	−1.70	0.026
65	Btrc	beta-transducin repeat containing protein	−1.77	0.030
66	E2f4	E2F transcription factor 4	−1.60	0.031
71	Ikbke	inhibitor of kappaB kinase epsilon	−2.04	0.038
73	Casp8	caspase 8	−1.51	0.039
74	Ripk2	receptor (TNFRSF)-interacting serine-threonine kinase 2	−1.47	0.040
75	Card11	caspase recruitment domain family, member 11	−1.50	0.041
76	Traf3	Tnf receptor-associated factor 3	−2.69	0.043
78	Tank	TRAF family member-associated NF-kappa B activator	−1.51	0.043
80	Rel	v-rel reticuloendotheliosis viral oncogene homolog (NFκB subunit)	−2.18	0.044
81	Wisp2	WNT1 inducible signaling pathway protein 2	−1.88	0.045
84	Btrc	beta-transducin repeat containing protein (an F-box protein)	−1.54	0.047

Analysis was as described in Table 1.

analysis on days 10, 14, and 21, and mineralization potential was assessed by combined von Kossa over ALP staining on days 14 and 21 and by alizarin red S (AR-S) staining on day 21. There was no significant difference in ALP staining or mineral apposition between wild-type versus AR-transgenic cultures without hormone addition at all time points. Thus, in the absence of hormone treatment, expression of the transgene does not influence osteoblast activity *in vitro*, consistent with the relative lack of phenotype in female transgenic mice *in vivo* (since females normally have low physiological concentrations of testosterone).

Effect of exogenous BMP2 on androgen inhibition of osteoblast differentiation

Based on bioinformatic analysis of the qPCR results, the TGF-β/BMP superfamily is a significant target for androgen action in bone (see Fig. 2). Given the impact of androgen on expression of several members of the BMP signaling cascade that could be expected to reduce BMP pathway activation, we chose to determine the consequences of BMP pathway activation on androgen-mediated inhibition of differentiation. Exogenous recombinant BMP2 was supplied with or without the nonaromatizable androgen DHT, and the effect on osteoblast differentiation in primary cultures was characterized. As shown in Fig. 4, ALP levels were affected by continuous treatment with vehicle, 10^{-8} M DHT, 100 ng/ml BMP2 or DHT combined with BMP2. In AR3.6-transgenic cultures, treatment with DHT significantly inhibited ALP activity by ~50% ($p < 0.05$). When exogenous BMP2 was supplied to activate BMP receptor signaling, BMP treatment alone significantly increased ALP activity ($p < 0.001$) as

expected. While there was no significant difference in ALP activity between combined DHT and BMP treatment compared with BMP treatment alone in either AR-transgenic genotype, a trend for inhibition was observed in AR3.6-transgenic cultures ($p = 0.08$ for BMP vs. combined treatment). Thus, inhibition of BMP signaling may be an important pathway by which DHT treatment inhibits osteoblast differentiation, since the negative effects of androgen can be attenuated with supplementation of exogenous BMP2 to drive the BMP receptor signaling pathway. These results suggest that BMP pathway activation is influenced by androgen signaling in bone and may contribute to androgen-mediated inhibition of osteoblast differentiation *in vivo*, but BMP signaling is likely not the only pathway altered.

Androgen inhibits both osteoblast proliferation and differentiation

Collectively, the bioinformatic analyses of the biological processes influenced by androgen signaling in cortical bone suggest that androgen inhibits osteoblast differentiation and function. To extend these results, we characterized the effects of DHT treatment in primary cultures during *in vitro* differentiation by analyzing the consequences of androgen administration throughout the course of osteoblast proliferation, matrix maturation and mineralization. Osteoblast proliferation was assessed by BrdU incorporation into DNA. Proliferating primary cultures of wild-type, AR3.6-transgenic and AR2.3-transgenic mice were treated continuously with vehicle or 10^{-8} M DHT for 5 days with BrdU incorporation during the final 20 h of culture. As shown in Fig. 5, the proliferation rate of osteoblasts treated with DHT was significantly decreased versus control cells in all
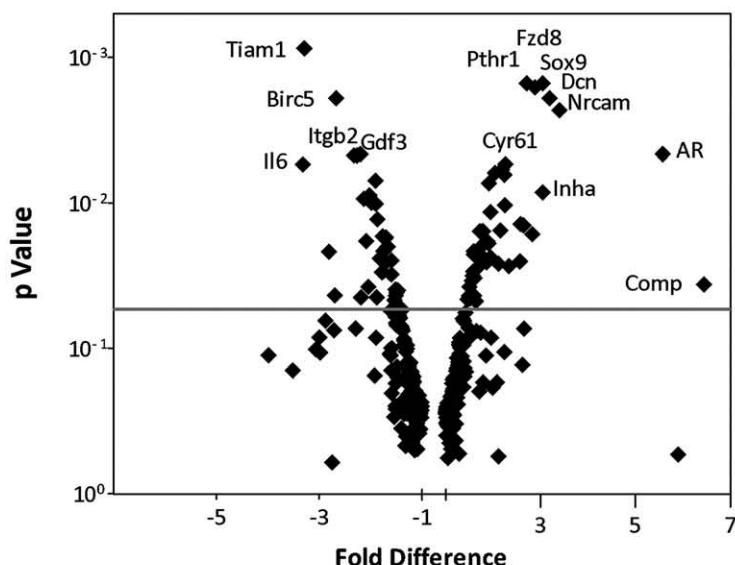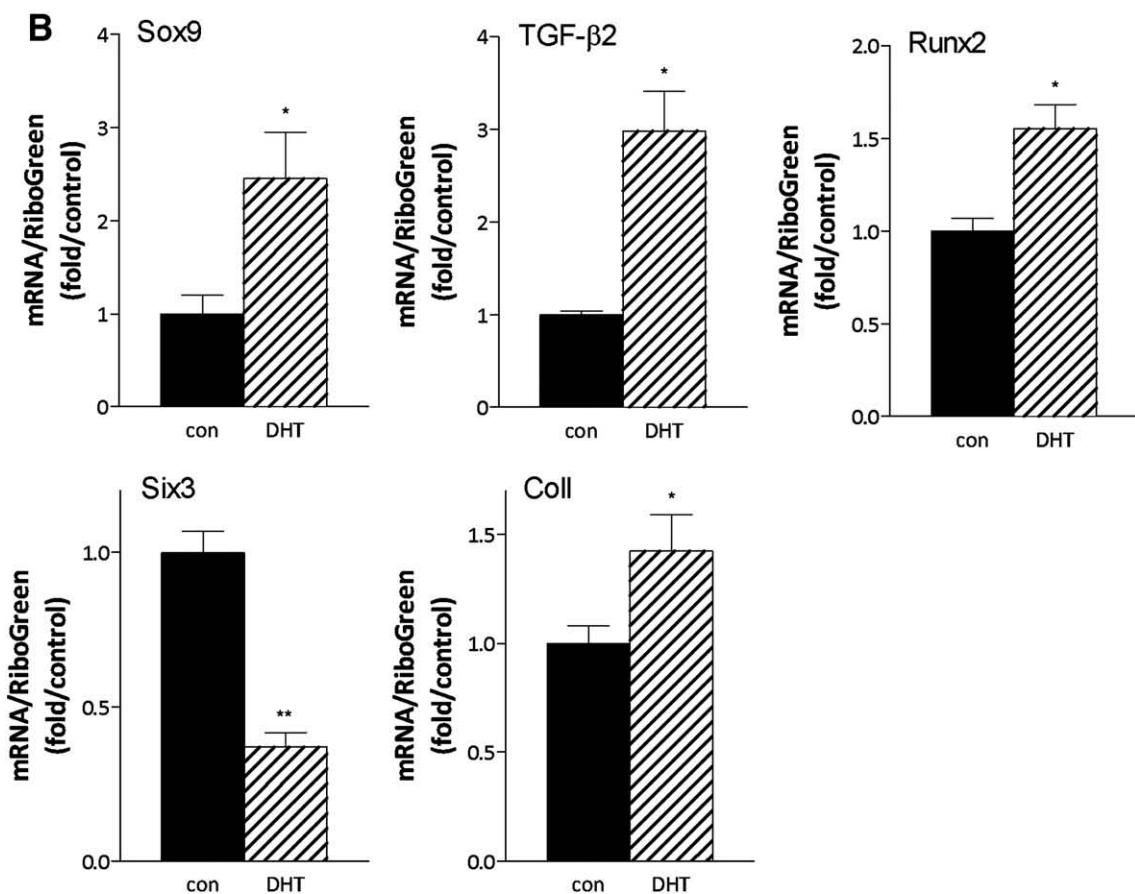
Fig. 1. Gene expression differences in AR3.6-transgenic cortical bone samples with qPCR confirmation of expression in primary osteoblast cultures. (A) A volcano plot of significant expression differences identified in AR3.6-transgenic bone. (B) Confirmation of selected genes identified in cortical bone samples by qPCR analysis in differentiated primary osteoblast cultures derived from AR3.6-transgenic mice treated continuously with 10^{-8} M DHT. Genes evaluated were Sox9, TGF-β2, Runx2, Six3 and collagen (Col). Data are expressed as mean ± SD ($n = 2$). *$p < 0.05$.

genotypes ($p < 0.05$ and 0.01). Thus, androgen treatment inhibits normal osteoblast proliferation, consistent with our previous report [16].

To then characterize the effects of androgen treatment on markers of osteoblast differentiation, we characterized ALP expression after treatment of cells with DHT (Fig. 6). Primary cultures from wild-type

and both AR-transgenic lines were treated continuously with vehicle or 10^{-8} M DHT in charcoal-stripped serum. ALP staining and activity were measured at day 14 during the matrix maturation stage of differentiation. The capacity of osteoblasts treated with DHT to differentiate was suppressed, shown by the reduction of ALP staining and significant inhibition of ALP activity in wild-type ($p < 0.01$) and
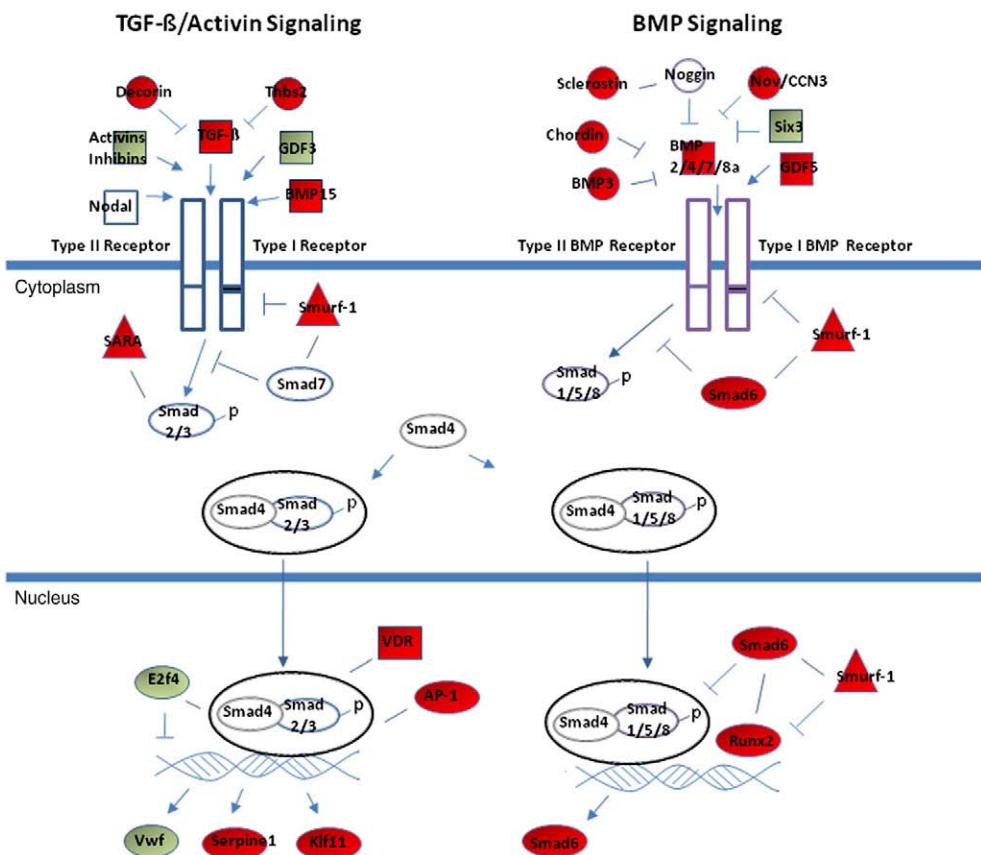
Fig. 2. Gene expression differences in TGF-β/BMP signaling in cortical bone from AR-transgenic males. Multiple components of BMP signaling are influenced by androgen action, with expression of several BMP antagonists up-regulated in bone. Up-regulated transcripts are shown in red; down-regulated are green; straight lines indicate protein binding or interaction; arrows indicate activation; inhibition is shown as a T-shaped line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

both AR-transgenic cultures ($p < 0.001$ and $p < 0.01$). The greatest inhibition of ~50–60% was observed in AR3.6-transgenic cultures.

We next evaluated the consequence of androgen treatment on mineralization in primary cultures treated with vehicle or 10^{-8} M DHT in charcoal-stripped serum. DHT was added continuously (0–21), or treatment was delayed until after cultures were confluent at day 10 (10–21) to assess the effects of androgen in more mature cells. Cultures treated with DHT demonstrated inhibition of mineralization assessed by alizarin red-S staining (Fig. 7; left panel). Quantitation of accumulated mineral (right panel) showed significant inhibition by DHT treatment at both time points ($p < 0.001$ vs. vehicle control) in wild-type and both AR-transgenic cultures. When androgen was present only during the last 10 days in more mature cultures, mineralization was also reduced ($p < 0.001$ vs. vehicle control) in wild-type and both AR-transgenic cultures. In AR3.6-transgenic cultures, DHT inhibition was significantly less in more mature cultures (d10–21) than that observed with continuous treatment ($p < 0.01$ vs. continuous treatment), illustrating the importance of inhibition during proliferation.

We then determined the effect of androgen treatment on mineral nodule formation in primary cultures from wild-type and both AR-transgenic lines. Cultures were treated continuously with vehicle or 10^{-8} M DHT for 21 days and differentiation was characterized by both ALP and von Kossa silver staining. Bone nodules were visualized by light microscopy and quantified from digital photographs, shown in Fig. 8. In agreement with inhibition of mineral accumulation assessed by alizarin red, nodule formation was significantly inhibited by DHT treatment in all three groups ($p < 0.05$ in AR2.3-transgenic cultures; $p < 0.001$ in both wild-type and AR3.6-transgenic cultures). The maximum inhibitory effect was seen in AR3.6-transgenic cells, with

over 60% reduction in nodule number. Thus, the inhibitory effect of androgen on osteoblast differentiation was observed for ALP activity at day 14 during matrix maturation, and at day 21 during mineralization and nodule formation. Combined, these observations confirm the identified biological processes as targets of androgen action in primary osteoblasts, and the negative consequences direct androgen signaling in bone. Results also suggest that inhibition of BMP signaling by androgen, as identified in array analyses, may play an important role in the detrimental effects observed.

Discussion

The effects of the steroid hormone androgen are pervasive, influencing many organ systems in the body including bone. We have previously shown that the direct effects of androgen in the skeleton are complex and envelope-specific, and both stimulation and inhibition of bone formation are observed *in vivo*. These studies, taken together, showed that AR signaling in mature osteoblasts/osteocytes *in vivo* inhibits endocortical bone formation and has detrimental consequences on matrix quality, osteoblast vigor and whole bone strength [6,7]. However, the cellular and molecular mechanisms through which androgen influences osteoblast function in this negative fashion *in vivo* remain uncharacterized. In order to identify signal transduction pathways that are affected by androgen signaling *in vivo*, mice with skeletally targeted AR overexpression were used as a source of endocortical bone samples for transcriptional profiling employing focused qPCR array analysis. This is the first study to characterize androgen-mediated inhibition of bone formation through molecular signaling *in vivo*, and has identified TGF-β/BMP/Activin pathways as central signal transduction cascades influenced
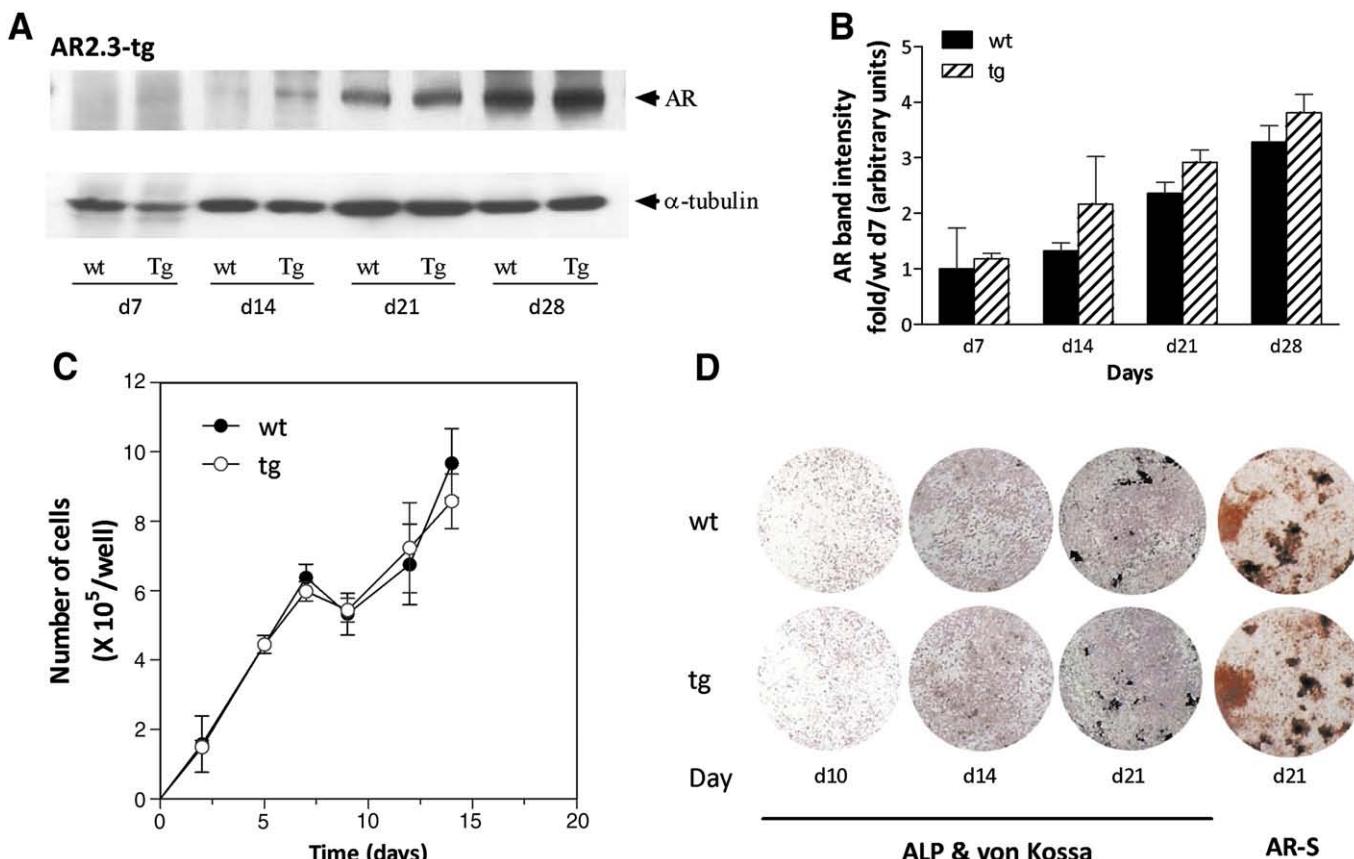
Fig. 3. Increased AR levels occur with osteoblast differentiation and with transgene expression but no effect of transgene expression on osteoblast growth or differentiation in the absence of exogenous androgen. AR abundance was determined in primary osteoblast cultures derived from wild-type littermate control or AR2.3-transgenic mice. Cell extracts were analyzed by Western blotting using polyclonal anti-AR antibody. (A) Time course analysis of AR protein levels. The upper panel shows AR levels observed in cell lysates of wild-type (wt) and AR2.3-transgenic calvarial osteoblasts (Tg) at indicated time points. The typical AR is indicated by the *arrow* at 110 kDa. The lower panel shows lysates reprobed with α-tubulin antibody in order to assess consistency of protein loading. Increased AR levels are seen over time during *in vitro* differentiation of primary calvarial cultures. (B) Quantitative analysis of AR abundance was performed by volume densitometry of scanned films. Data are presented as protein/α-tubulin ratio normalized to expression values of wild-type at day 7. Data are expressed as mean ± SD. Analysis of increased AR levels revealed an extremely significant effect of time ($F = 46.53$; $p < 0.0001$) and a very significant effect of genotype ($F = 11.44$; $p < 0.001$), with no interaction. The highest levels of AR are present in cultures of mature osteoblasts and osteocytes. (C) Growth characteristics of untreated wild-type and AR2.3-transgenic osteoblasts. Cells were counted at indicated time points. Data are expressed as mean ± SD ($n = 3$). (D) Differentiation potential of untreated wild-type and AR2.3-transgenic osteoblasts. Cultures were stained for ALP expression on days 10, 14, and 21 by histochemical analysis, and mineralization potential was assessed by von Kossa over ALP staining on days 14 and 21 and by alizarin red S (AR-S) staining on day 21. Data are expressed as mean ± SD ($n = 3$). There were no statistical differences in ALP staining or mineral apposition between wild-type and AR2.3-transgenic osteoblastic cells in the absence of hormone.

by AR in endocortical bone. Bioinformatic analyses revealed that osteoblast proliferation, differentiation and mineralization were major biological targets affected by androgen in bone. To corroborate these observations, normal primary cultures derived from wild-type and from AR-transgenic mice were characterized. We show that exogenous BMP2 treatment can reduce inhibition of osteoblast differentiation after androgen treatment. We also show that androgen treatment directly inhibits normal osteoblast proliferation, differentiation and mineralization in a cell autonomous fashion, confirming bioinformatic analyses.

The negative effects of androgen on endocortical bone formation may play a role in limiting the anabolic response to androgen *in vivo*. Identification of the signal transduction pathways that are influenced by androgen in this compartment may thus provide useful information for drug development. Cortical bone is an important therapeutic target, comprising approximately 70% of bone mass. Cortical bone is also the site of fractures with the highest morbidity and mortality. Analysis of AR-transgenic mice has shown that with the exception of a positive effect of androgen at the periosteal surface, the effects of androgen on cortical bone quality and strength are detrimental [6,7]. Bioinformatic analyses of expression differences between wild-type and transgenic cortical bone samples identified osteoblast proliferation and differentiation as important biological themes impacted by

androgen signaling. This was not unexpected, given that both AR-transgenic lines showed a low turnover phenotype, with a significant reduction in osteoblast vigor in cortical bone samples and a significant reduction in bone formation on the endocortical surface [6,7]. This accumulated evidence does not support the concept of strong anabolic responsiveness as a consequence of androgen therapy directly in long bone in the endocortical compartment. Positive anabolic effects of androgen in bone may thus be limited to distinct lineages, for example immature cells in the periosteal compartment [6,7] or indirect effects mediated by changes in muscle mass after systemic androgen therapy.

As indicated in bioinformatic analyses, the effects of androgen on osteoblast proliferation and function *in vitro* were inhibitory; including reduced ALP activity, reduced mineralization and reduced mineral nodule formation. In aggregate, these data help resolve controversies in the literature regarding the effects of androgen on osteoblast function. DHT treatment inhibited normal osteoblast proliferation, consistent with previous reports of inhibition of both mitogenic signaling and MAP kinase activity [16] and enhancement of osteoblast apoptosis [21] by chronic DHT treatment. Although mineral accumulation and nodule formation was inhibited by DHT treatment in all three culture models, previous reports had suggested that androgen treatment increased mineralization *in vitro* [18,20,23,24]. These differences may be attributed to treatment time and conditions,
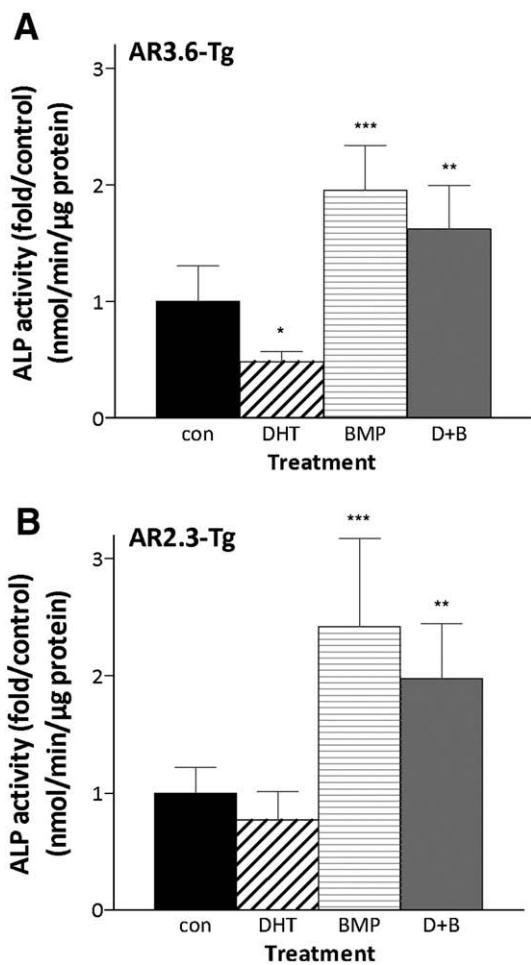
Fig. 4. Reversal of androgen inhibition of osteoblast differentiation by exogenous BMP2. Primary osteoblasts were treated with vehicle, DHT (10^{-8} M), BMP2 (100 ng/ml) or DHT plus BMP2, based on altered BMP signaling shown in Fig. 2. Changes in ALP were measured after continuous treatment for 14 days. (A) Primary cultures from AR3.6-transgenic mice ($n = 6$). (B) AR2.3-transgenic calvarial osteoblasts ($n = 6$). Data are expressed as mean ± SD. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ compared to vehicle.
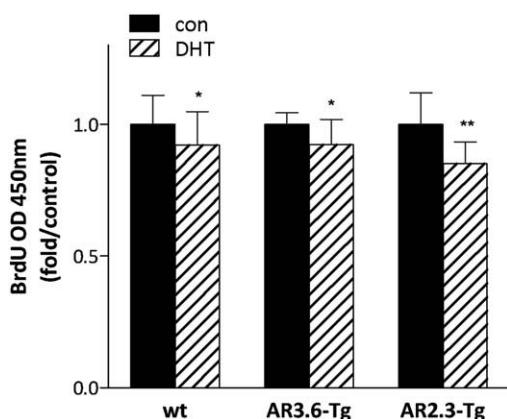
Fig. 5. Androgen-mediated inhibition of osteoblast proliferation. Primary osteoblasts were cultured from wild-type, AR3.6-transgenic and AR2.3-transgenic mice for 5 days in the absence or presence of DHT (10^{-8} M). Changes in proliferation were measured using BrdU incorporation. Cultures were grown in 96-well dishes and pulsed for 20 h with BrdU. Measurements were performed for each genotype or treatment and expressed as fold vs. vehicle control. Data are expressed as mean ± SD ($n = 12$–24). DHT significantly reduced osteoblast proliferation in wild-type, AR3.6-transgenic and AR2.3-transgenic cultures. *$p < 0.05$; **$p < 0.01$ compared to vehicle.

and the various culture models employed. The inhibition of mineralization and nodule formation shown in vitro in this study is consistent with previous in vivo analysis, with reduced bone matrix mineral accumulation in AR2.3-transgenic males [7]. Thus, the cell autonomous effect of androgen inhibition of osteoblast function was confirmed in three primary culture models. It was useful to characterize these responses in vitro since inhibition of bone formation in vivo, as was observed in both AR3.6-transgenic and AR2.3-transgenic males, is not always linked with inhibition of in vitro differentiation. For example, Zfp521 overexpression is associated with inhibition/delay of differentiation in vitro but promotion of bone formation in vivo [29]. Thus, from a mechanistic perspective, these studies identify cell autonomous direct actions in osteoblasts that likely underlie the response to androgen signaling in vivo. In endocortical bone, the compartment-specific effects of androgen that have been observed in vivo are thus likely mediated (in part) through inhibitory action throughout the osteoblast lineage, including direct inhibitory effects on the proliferation, matrix maturation and mineralization stages of differentiation.

A minority of the specific genes identified in the qPCR analysis of AR-transgenic bone have been previously characterized as regulated by androgen in bone or in other tissues. We searched for androgen-controlled genes using Gene Set Enrichment Analysis (GSEA) [30]. However, there were no overlapping genes with the Nelson_Androgen_Up (Or _Dn), nor was there overlap with another GSEA group "Androgen_Genes". We also searched using the Androgen-Responsive Gene Database (ARGDB) [31]. Of the 78 genes significantly regulated, 16 genes (21%) were identified in the database. Finally, PubMed searches identified several genes that are regulated by androgen, including up-regulation of TGF-β2 protein in osteoblasts [32]; inhibition of inhibin A in prostate [33]; inhibition of Sox9 seen in a model of muscle development [34] but an increase of Sox9 in prostate [33]. The most up-regulated transcripts were Casr, Nov (CCN3), Bmp15, Comp and Gdf5 while the most down-regulated transcripts were MCHR 1, neurogenin 1, inhibin-β C, interleukin-6 and Traf3. BMP15, Comp and Gdf5 are all important in TGF-β/BMP signaling. CCN3 exerts inhibitory effects on BMP-2-induced osteoblast differentiation and impairs Wnt signaling. Interleukin-6 and Traf3 are important in NFκB signaling. Selected differences in expression identified in the qPCR array were confirmed in vitro, suggesting that at least some of the expression differences identified in vivo are a consequence of a direct cell autonomous response to androgen through androgen receptor signaling in osteoblasts, and not as a consequence of biomechanical adaptations to changes in bone geometry or changes in other cells/tissues/organs including osteoclasts.

Bioinformatic analyses of the expression profile differences identified TGF-β superfamily pathways as major androgen targets in endocortical bone. TGF-β is a multifunctional secreted growth factor that regulates a wide variety of cellular processes, including proliferation, differentiation and matrix synthesis. Two main branches of TGF-β signaling have been described, with TGF-β/Activin/Nodal ligands that activate transcription factors Smad2 and Smad3, while BMP/GDF ligands activate Smad1, Smad5, and Smad8 [35]. Inhibitory Smad (I-Smad) proteins are Smad6 and Smad7. Both major branches of TGF-β signaling were significantly affected by androgen action in bone (see Fig. 2). It should be noted however, that focused arrays only characterize pathways that are hypothesized to be involved in the phenomena under evaluation. In this analysis we targeted pathways associated with bone formation because a major phenotype observed in both AR-transgenic lines was inhibition of endocortical bone formation. To identify novel targets, additional studies with genome-wide analysis will be required.

Constitutive activation of BMP signaling in mature osteoblasts has been shown to enhance bone formation and osteoblast differentiation [36], while inhibition of BMP expression in a variety of models can reduce bone formation [37–39]. Negative regulation of BMP signaling
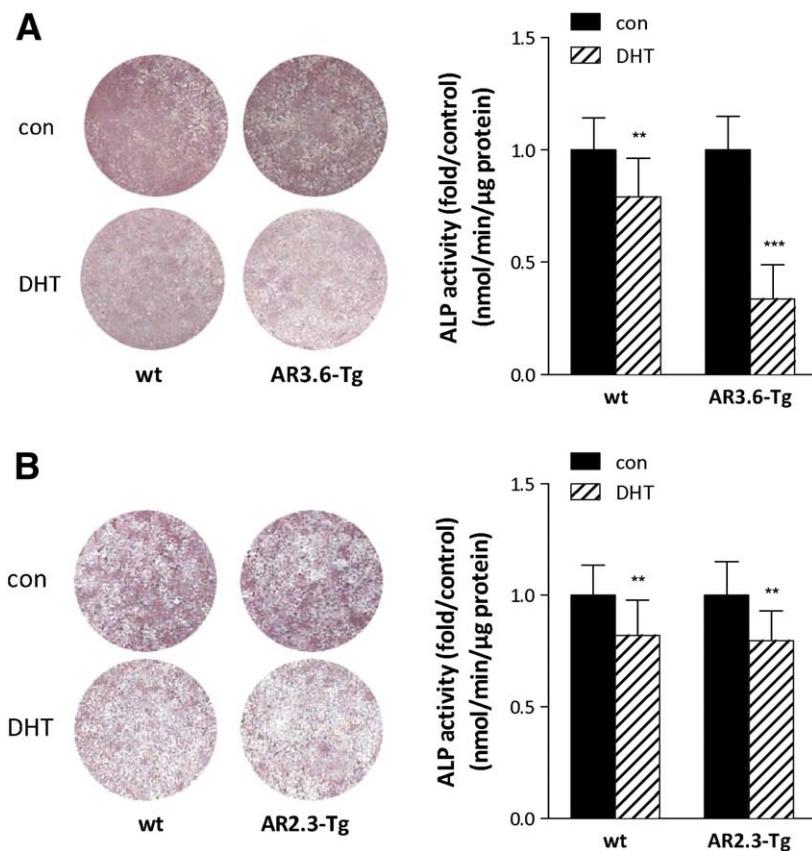
Fig. 6. Androgen-mediated inhibition of ALP activity during *in vitro* differentiation in primary cultures. Primary osteoblasts were cultured in the absence or presence of DHT (10^{-8} M). Changes in ALP activity were measured after DHT treatment for 14 days in wild-type, AR3.6-transgenic and AR2.3-transgenic calvarial osteoblasts. (A) Primary cultures from AR3.6-transgenic mice ($n = 11$–12). (B) Primary cultures from AR2.3-transgenic mice ($n = 12$). ALP staining is shown on left and ALP activity on right; ALP activity was expressed as fold vs. vehicle control. Data are shown as mean $\pm$ SD. A significant decrease in ALP activity was demonstrated with DHT treatment. $**p < 0.01$; $***p < 0.001$ compared to vehicle.

pathways was a specifically enriched GO category identified in our bioinformatic analysis. BMPs bind and activate two serine/threonine kinase receptors, the type II (BMPRII) and the type I receptors. These form a heteromeric signaling complex in the presence of ligand, where the type II receptor phosphorylates the type I receptors which then activates signaling by intracellular effector Smad proteins. Smurf1 reduces BMP signaling by promoting Smad1 and Smad5 ubiquitination and degradation. Given the observed increase in Smurf1 expression, overall BMP pathway activation is likely reduced by androgen action to some extent. Sox9 levels were increased, consistent with a transient increase in Sox9 expression in bone tissue after BMP2 inhibition [37]. In addition, BMP antagonist chordin expression was up-regulated in AR-transgenic samples. Although chordin levels increase as osteoblasts differentiate, exogenous chordin inhibits differentiation and mineralization [40]. In addition, inhibitory BMP3 levels were increased by androgen action. BMP3 antagonizes BMP2 signaling and is an inhibitor of osteogenesis *in vitro* and of bone formation *in vivo* [41]. Similarly, CCN3/NOV antagonizes BMP-2 signaling [42], and CCN3/NOV expression was increased in transgenic bones. Given these multiple expression differences, it appears that aspects of BMP pathway activation are reduced as a consequence of androgen receptor signaling in endocortical bone. Data presented here demonstrates that exogenous BMP2 treatment can at least partially overcome androgen inhibition of osteoblast differentiation. To our knowledge, this is the first report of androgen regulation of BMP signaling in bone tissue.

The second branch of TGF-β signaling involving activin/inhibin signaling was also differentially regulated in transgenic bones. Inhibins and inhibitory BMP3 compete for binding to the type II activin receptors to act as antagonists of activin signaling. Of interest, results from the qPCR arrays showed increased expression of the inhibin α-subunit and BMP3 with down-regulation of the inhibin β-subunit C in endocortical bone samples from male transgenic mice. Elevated α-subunit expression would favor the formation of heterodimers (inhibin α/βA or α/βB), thus leading to suppression of activin signaling. In addition, the decrease in βC expression may increase bioactive inhibin A levels because of reduced formation of activin AC (in the presence of higher α-subunit expression). In mouse bone marrow cultures, inhibins were shown to suppress while activins stimulated osteoblastogenesis and osteoclastogenesis [43]. Activin receptors are also up-regulated during the process of distraction osteogenesis [44]. Suppression of osteoblastogenesis by inhibins is consistent with our observations *in vivo* in both AR-transgenic lines. It is also possible that the decreased ALP staining and activity, and reduced mineralization *in vitro*, may be associated with increased inhibin signaling. However, there remains some debate as to the exact role of activin/inhibins in bone mineral homeostasis that may relate to the length of inhibin exposure [45]. Varying results may also reflect distinct consequences of activin/ inhibin signaling in discrete bone compartments or at different times of development. In adult AR-transgenic mice, increased inhibin (and reduced activin) signaling is associated with reduced endocortical bone formation and reduced mineralization. Finally, inhibitory Smad6 expression was increased by androgen action, which would likely reduce activin receptor-like kinase (ALK) signaling. Smad6 inhibits signaling from the ALK-3/6 subgroup, in preference to that from the ALK-1/2 or ALK-4/5/7 subgroup of BMP type I receptors [46]. Notably, Smad6 inhibited osteogenic differentiation *in vitro*
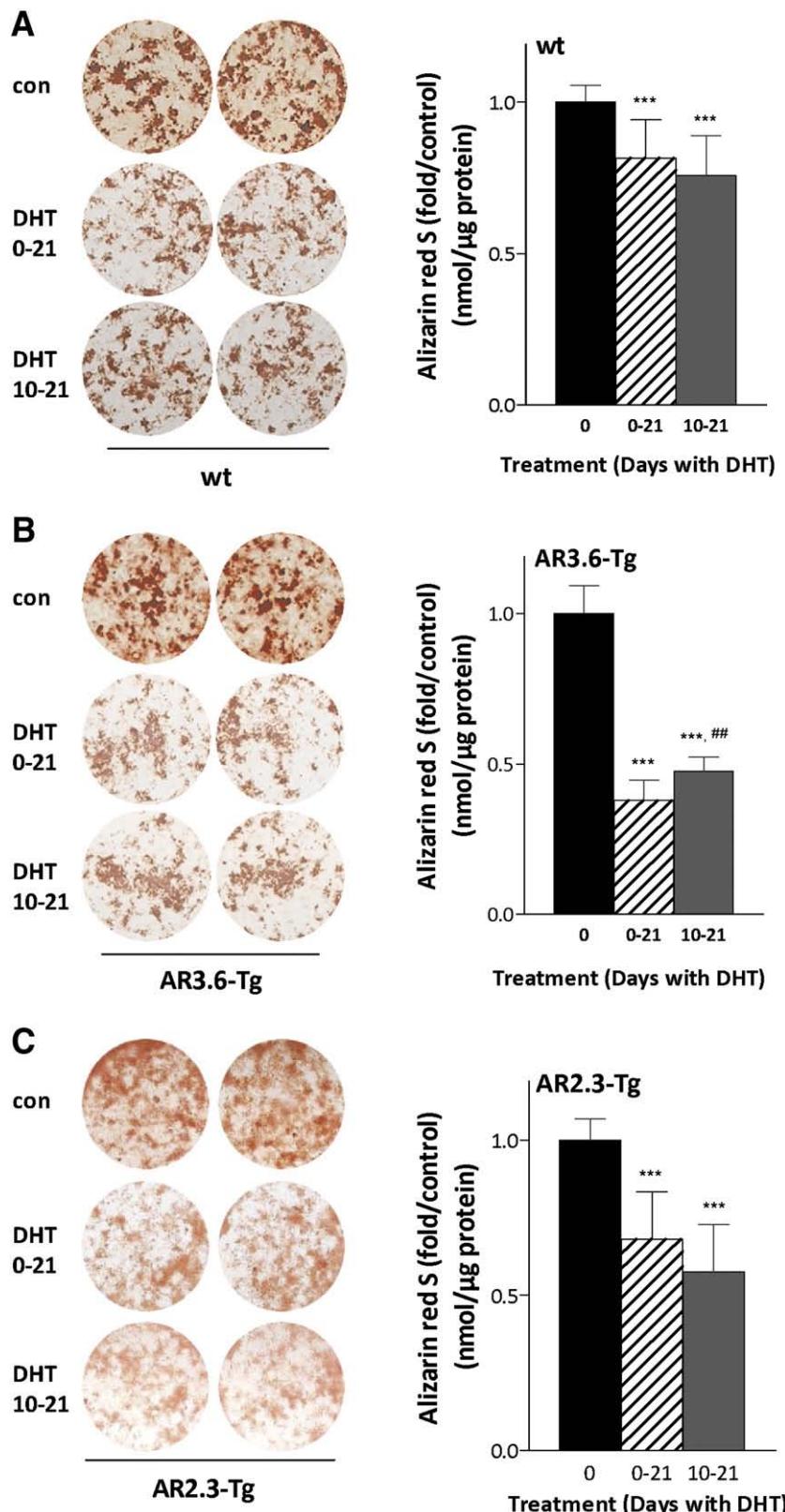
Fig. 7. Inhibition of osteoblast mineral deposition after DHT treatment in primary cultures from wild-type and AR-transgenic mice. Osteoblast-enriched primary cultures were derived from fetal calvaria by collagenase digestion. Cells were grown with or without DHT (10^{-8} M) added on the indicated treatment days and isolated on day 21, and mineralization was assessed by alizarin red-S (AR-S) staining and AR-S quantitation after extraction. (A) Primary cultures from wild-type mice ($n = 12$). (B) Primary cultures from AR3.6-transgenic mice ($n = 9$). (C) Primary cultures from AR2.3-transgenic mice ($n = 9$). AR-S staining is shown on left and AR-S accumulation on right; AR-S accumulation was expressed as fold vs. vehicle control. All data are shown as mean ± SD. Analysis of AR-S levels revealed an extremely significant interaction ($F = 26.88$; $p < 0.0001$) between genotype and treatment. A highly significant decrease in mineral accumulation was observed with DHT treatment in all treatment groups. ***$p < 0.001$ compared to vehicle; ##$p < 0.01$ compared to continuous treatment 0–21 days.

A ALP & von Kossa
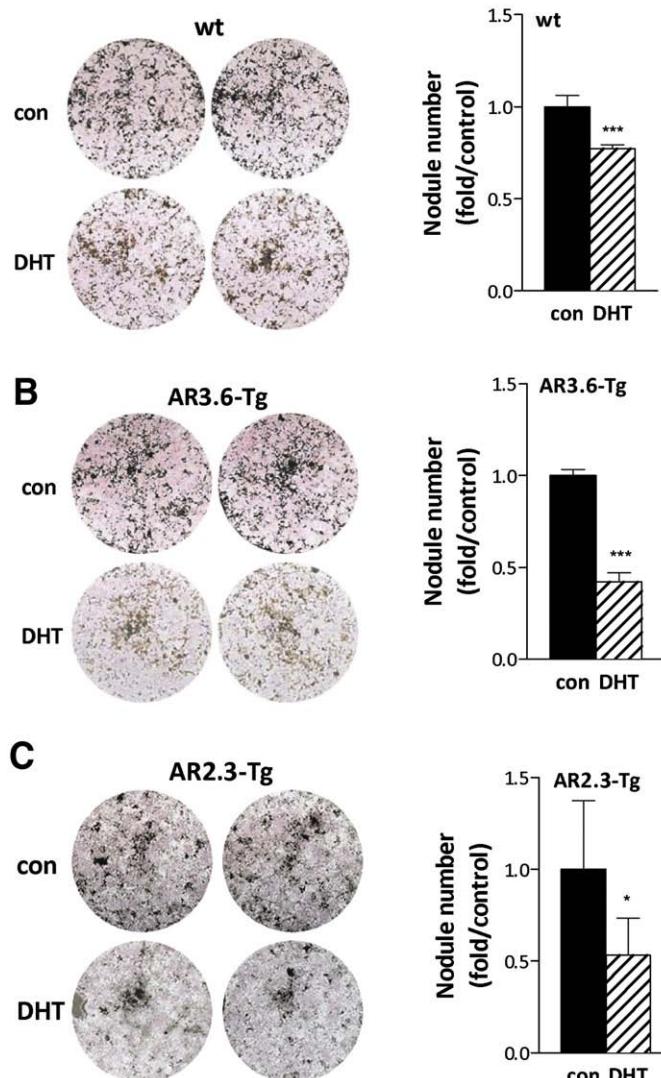
Fig. 8. Von Kossa silver staining of mineralized bone nodules over ALP histochemical analysis. Calvarial osteoblasts were cultured with or without DHT (10^{-8} M) for 21 days. The cultures were evaluated by ALP histochemistry and von Kossa silver staining on left. Quantification of bone nodules formed is shown on right. (A) Primary cultures from wild-type mice ($n = 5$). (B) Primary cultures from AR3.6-transgenic mice ($n = 5$). (C) Primary cultures from AR2.3-transgenic mice ($n = 4$–5). Representative images for combined ALP and von Kossa staining are shown. Results are expressed as mean ± SD. A significant decrease in the number of bone nodules was observed with DHT addition. $*p < 0.05$; $***p < 0.001$ compared to vehicle.

[47], and reduced induction of Smad6 is associated with increased bone formation and osteosclerosis in vivo [48].

In addition to changes in inhibin/activin signaling, results also suggest modulation of TGF-β signaling in transgenic endocortical bone. Results from qPCR analysis showed increased TGF-β2, Smad anchor for receptor activation (SARA) and Runx2 gene expression. SARA recruits and presents Smad2/3 to the receptor complex, facilitating TGF-β and activin/nodal signaling. SARA does not bind Smad1 and thus does not also influence BMP signaling [49]. An activated Smad complex may interact with Runx2 and Sox9, resulting in changes in transcriptional regulation of target genes (see [50]). Runx2 also physically associates with AR, and hormone binding of AR can abrogate Runx2 binding to DNA [51]. Interestingly, recent reports have shown that reductions in TGF-β signaling at the level of TGF-β1 result in a low bone mass phenotype [52], and also highlight the importance of androgen inhibition of TGF-β signaling in myogenic

differentiation [53]. Taken together, cross-talk among these various TGF-β intracellular pathways and integration of the downstream signaling events may help define the bone-specific response to androgen.

There are a variety of reports that suggest androgens are capable of strongly suppressing osteoclastogenesis, both through a direct effect on osteoclasts [54] and indirect effects on osteoblasts [55]. Notably, the interleukin/NFκB pathway was identified in our bioinformatic analyses of the expression profile differences as a target for androgen in cortical bone, and osteoclast differentiation as a biological target (see Supplemental Figure 1). The qPCR array results also showed increased expression of osteoprotegerin, and inhibition of interleukin-6, interleukin-1α and receptor activator of NFκB (RANK) expression; combined these changes are expected to reflect inhibition of osteoclastogenesis and activity. This is consistent with the in vivo response observed in AR3.6-transgenic mice, where OPG expression was increased [6]. In addition, both TRAPb5c and cross-linked C-telopeptide of type I collagen (CTx-I) levels are reduced in transgenic males [7], consistent with a reduction in osteoclast activity. Finally, the trabecular phenotype of reduced spacing and increased trabecular number observed in both AR-transgenic lines [6,7] is a hallmark of anti-resorptive responses. Our results suggest that indirect regulation of osteoclasts, mediated in part by osteoblasts with targeted AR overexpression, plays a role in inhibition of osteoclast activity through modulation of the NFκB/ interleukin pathway.

In conclusion, expression profile differences were characterized using array-based qPCR analysis of endocortical bone from wild-type and AR-transgenic males. Bioinformatics identified the TGF-β super-family with BMP signaling as a major pathway among those associated with bone formation to be significantly altered by androgen in vivo, and also revealed proliferation, osteoblast differentiation and mineralization as biological processes affected. Stimulation of BMP signaling with exogenous BMP2 can partially abrogate androgen inhibition of differentiation. In addition, nonaromatizable DHT inhibited osteoblast proliferation, differentiation and indices of mineralization including mineral accumulation and mineralized nodule formation in normal primary cultures from wild-type and both AR-transgenic lines in a cell autonomous fashion, consistent with in vivo array data and bioinformatic analyses. Thus, androgen action directly suppresses osteoblast function throughout the lineage. Such detrimental effects on osteoblast function in endocortical bone would be expected to compete with the anabolic bone-formation activity seen at periosteal sites, and thus may underlie the generally disappointing results of androgen therapy. Finally, these data have therapeutic implications for the continued development of anabolic drugs for the treatment of osteoporosis, with identification important androgen-mediated signaling pathways that may influence bone quality.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bone.2009.10.039.

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Androgens and Skeletal Biology: Basic Mechanisms

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I. INTRODUCTION

The obvious impact of the menopause on skeletal health has focused much of the research describing the general action of gonadal steroids on the specific effects of estrogen in bone (see Chapter 14). However, androgens clearly have important beneficial effects, in both men and women, on skeletal development and on the maintenance of bone mass. Thus it has been demonstrated that androgens (a) influence growth plate maturation and closure helping to determine longitudinal bone growth during development, (b) mediate regulation of trabecular (cancellous) and cortical bone mass in a fashion distinct from estrogen, leading to a sexually dimorphic skeleton, (c) modulate peak bone mass acquisition, and (d) inhibit bone loss [1]. In castrate animals, replacement with nonaromatizable androgens (*e.g.* 5α-dihydrotestosterone, DHT) yields beneficial effects that are clearly distinct from those observed with estrogen replacement [2, 3]. In intact females, blockade of the androgen receptor (AR) with the specific AR antagonist hydroxyflutamide results in osteopenia [4]. Furthermore, treatment with nonaromatizable androgen alone in females results in improvements in bone mineral density [5]. Finally, combination therapy with estrogen and androgen in post-menopausal women is more beneficial than either steroid alone [6-8], indicating non-parallel and distinct pathways of action. Combined, these reports illustrate the distinct actions of androgens and estrogens on the skeleton. Thus, in both men and women it is probable that androgens and estrogens each have important yet distinct functions during bone development, and in the subsequent maintenance of skeletal homeostasis in the adult. With the awakening awareness of the importance of the effects of androgen on skeletal homeostasis, and the potential to make use of this information for the treatment of bone disorders, much remains to be learned.

II. ANDROGENS AND THE ROLE OF ANDROGEN METABOLISM

A. Metabolism of Androgens in Bone: 5α-Reductase, Aromatase and 17β-hydroxysteroid Dehydrogenase Activities

Sex steroids, ultimately derived from cholesterol, are synthesized predominantly in gonadal tissue, the adrenal gland and placenta as a consequence of enzymatic conversions. After peripheral metabolism, androgenic activity is represented in a variety of steroid molecules that include testosterone (Fig. 1). There is evidence in a range of tissues that the eventual cellular effects of testosterone may not be the result (or not only the result) of direct action of testosterone, but may also reflect the effects of sex steroid metabolites formed as a consequence of local enzyme activities. The most important testosterone metabolites in bone are 5α-DHT (the result of 5α reduction of testosterone) and estradiol (formed by the aromatization of testosterone). Testosterone and DHT are the major and most potent androgens, with androstenedione (the major circulating androgen in women) and dehydroepiandrosterone (DHEA) as immediate androgen precursors that exhibit weak androgen activity [9]. In men, the most abundant circulating androgen metabolite is testosterone while concentrations of other weaker androgens like androstenedione and DHEA-sulfate are similar between males and females. Downstream metabolites of DHT and androstenedione are inactive at the AR, and include 5α-androstane-3α or 3β,17β-diol (3α/β-androstanediol) and 5α-androstanedione. Data suggests that aromatase cytochrome P450 (the product of the CYP19 gene), 17β-hydroxysteroid dehydrogenase (17ß-HSD), and 5α-reductase activities are all present in bone tissue as described below, at least to some measurable extent in some compartments, but the biologic relevance of each remains somewhat controversial.

-- Insert Figure 1 here --

5α-reductase is an important activity with regard to androgen metabolism in general, since testosterone is converted to the more potent androgen metabolite DHT via 5α-reductase

action [10]. 5α-reductase activity was first described in crushed rat mandibular bone [11] with similar findings reported in crushed human spongiosa [12]. Two different 5α-reductase genes encode type 1 and type 2 isozymes in many mammalian species [13]; human osteoblastic cells express the type 1 isozyme [14]. Essentially the same metabolic activities were reported in experiments with human epiphyseal cartilage and chondrocytes [15]. In general, the K_m values for bone 5α-reductase activity are similar to those in other androgen responsive tissues [12, 16]. However, the cellular populations in many of these studies were mixed and hence the specific cell type responsible for the activity is unknown. Interestingly, Turner *et al.* found that periosteal cells do not have detectable 5α-reductase activity [17], raising the possibilities that the enzyme may be functional in only selected skeletal compartments, and that testosterone may be the active androgen metabolite at this clinically important site.

From a clinical perspective, the general importance of this enzymatic pathway is uncertain, as patients with 5α-reductase type 2 deficiency have normal bone mineral density [18] and Bruch *et al.* found no significant correlation between enzyme activities and bone volume [10]. In mutant null mice lacking 5α-reductase type 1 (mice express very little type 2 isozyme), the effect on the skeleton has not been analyzed due to midgestational fetal death as a consequence of estrogen excess [19]. Analysis of the importance of 5α-reductase activity has been approached with the use of finasteride (an inhibitor of 5α-reductase activity); treatment of male animals does not recapitulate the effects of castration [20], strongly suggesting that reduction of testosterone to DHT by 5α-reductase is not the major determinant in the effects of gonadal hormones on bone. Consistent with this finding, testosterone therapy in hypogonadal older men, either when administered alone or when combined with finasteride, increases bone mineral density, again suggesting that DHT is not essential for the beneficial effects of testosterone on bone [21]. Thus, the available clinical data remains uncertain, and the impact of this enzyme, which isozyme may be involved, whether it is uniformly present in all cell types

involved in bone modeling/remodeling, or whether local activity is important at all, remain unresolved issues.

Another important enzymatic arm of testosterone metabolism involves the biosynthesis of estrogens from androgen precursors, catalyzed by aromatase. Of note, this enzyme is well known to be both expressed and regulated in a very pronounced tissue-specific manner [22], and also demonstrates species differences, given the low levels in mice. Modest levels of aromatase activity have been reported in bone from mixed cell populations derived from both sexes [23-25] and from osteoblastic cell lines [16, 26, 27]. Aromatase expression in intact bone has also been documented by *in situ* hybridization and immunohistochemical analysis [25]. Aromatase mRNA is expressed predominantly in lining cells, chondrocytes and some adipocytes, however there is no detectable expression in osteoclasts, or in cortical bone in mice [28]. At least in vertebral bone, the mesenchymal distal promoter I.4 is predominantly utilized [29]. The enzyme kinetics in bone cells seem to be similar to those in other tissues, although the V_{max} may be increased by glucocorticoids [27]. Whether the level of aromatase activity in bone is high enough to produce physiologically relevant concentrations of steroids remains an open question; nevertheless in the male only 15% of circulating estrogen is produced in the testes, with the remaining 85% produced by peripheral metabolism that could include bone as one site of conversion [30].

Aromatase catalyzes the metabolism of adrenal and testicular C19 androgens (androstenedione and testosterone) to C18 estrogens (estrone and estradiol), thus producing the potent estrogen estradiol (E2) from testosterone, and the weaker estrogen estrone (E1) from its adrenal precursors androstenedione and DHEA [23]. Typically in the circulation, E2 will make up to 40 percent of total estrogen, E1 will make up an additional 40 percent, with estriol (E3) comprising the remaining 20 percent of total estrogen [31]. In addition to aromatase itself, osteoblasts contain enzymes that are able to inter-convert estradiol and estrone (17β-HSD), and to hydrolyze estrone sulfate, the most abundant estrogen in the circulation, to estrone (steroid

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sulfatase) [26, 32]. Nawata *et al.* have reported that dexamethasone and $1\alpha,25(OH)_2D_3$ synergistically enhance aromatase activity and aromatase mRNA expression in human osteoblast-like cells [23]. In addition, both leptin and $1\alpha,25(OH)_2D_3$ treatment increased aromatase activity in human mesenchymal stem cells during osteogenesis, but not during adipogenesis [33]. Additional studies are needed to better define expression, given the potential importance of the enzyme, and its regulation by a variety of mechanisms (including androgens and estrogens) in other tissues [22, 34].

The clinical impact of aromatase activity and an indication of the importance of conversion of circulating androgen into estrogen is shown in reports of women and men with aromatase deficiencies, who present with a skeletal phenotype [35]. Interestingly, natural mutation is remarkably rare with only seven males and six females reported to date. The presentation of men with aromatase deficiency is very similar to that of a man with estrogen receptor-α (ERα) deficiency [36], namely an obvious delay in bone age, lack of epiphyseal closure and tall stature with high bone turnover and osteopenia [30], suggesting that aromatase (and likely estrogen action) has a substantial role to play during skeletal development in the male. In addition, estrogen therapy of males with aromatase deficiency has been associated with an increase in bone mass [30] particularly in the growing skeleton [37]. Inhibition of aromatization pharmacologically with nonsteroidal inhibitors (such as vorozole or letrozole) results in modest decreases in bone mineral density and changes in skeletal modeling in young growing orchidectomized males [38], and less dramatically so in boys with constitutional delay of puberty treated for one year [39], suggesting short term treatment during growth has limited negative consequences in males. Inhibition of aromatization in older orchidectomized males resembles castration with similar increases in bone resorption and bone loss, suggesting that aromatase activity likely plays a role in skeletal maintenance in males [40]. These studies herald the importance of aromatase activity (and estrogen) in the mediation of some androgen action in bone in both males and females. The finding of these enzymes in bone clearly raises

the difficult issue of the origin of androgenic effects in the skeleton; do they arise solely from direct androgen effects (as is suggested by the actions of nonaromatizable androgens such as DHT) or also from the local or other site production of estrogenic intermediates? The results described above would seem to indicate that both steroids appear to be important to both male and female skeletal health.

The 17β-HSDs (most of which are dehyrogenase-reductases, except type 5 that is an aldo-keto reductase) have been shown to either catalyze the last step of sex steroid synthesis or the first step of their degradation (to produce weak or potent sex steroids via oxidation or reduction, respectively), and can thus also play a critical role in peripheral steroid metabolism. The oxidative pathway forms 17-ketosteroids while the reductive pathway forms 17β-hydroxysteroids. The enzyme reversibly catalyzes the formation of androstenediol (an estrogen) from DHEA, in addition to the biosynthesis of estradiol from estrone, the synthesis of testosterone from androstenedione, and the production of DHT from 5α-androstanedione all via the reductive activity of 17β-HSD. Of the 13 enzyme isotypes of 17β-HSD activity [31], types 1-4 have been demonstrated in human osteoblastic cells [41].

The administration of testosterone can stimulate bone formation and inhibit bone resorption, likely through multiple mechanisms that involve both androgen and estrogen receptor-mediated processes. However, there is substantial evidence that some, if in fact not most, of the biologic actions of androgens in the skeleton are mediated by AR. Both *in vivo* and *in vitro* systems reveal the effects of the nonaromatizable androgen DHT to be essentially the same as those of testosterone (*vida infra*). In addition, blockade of the AR with the receptor antagonist flutamide results in osteopenia as a result of reduced bone formation [4]. In addition, complete androgen insensitivity results in a significant decrease in bone mineral density in spine and hip sites [18] even in the setting of strong compliance with estrogen treatment [42]. These reports clearly indicate that androgens, independent of estrogenic metabolites, have primary effects on osteoblast function. However, the clinical reports of subjects with aromatase

deficiency also highlight the relevance of metabolism of androgen to bio-potent estrogens at least in the circulation, to influence bone development and/or maintenance. It thus seems likely that further elucidation of the regulation steroid metabolism, and the potential mechanisms by which androgenic and estrogenic effects are coordinated, will have physiological, pathophysiological, and therapeutic implications.

B. Synthetic androgens

In addition to the endogenous steroid metabolites highlighted in Fig. 1, there are also a variety of drugs with androgenic activity. These include anabolic steroids, such as nonaromatizable nandrolone, that bind and activate AR (albeit with lower affinity than testosterone [43]), and a class of drugs under extensive development referred to as selective AR modulators (SARMs), that demonstrate tissue-specific agonist or antagonist activities with respect to AR transactivation [44]. These orally active nonsteroidal nonaromatizable SARMS are being developed to target androgen action in bone, muscle, fat and to influence libido but to not exacerbate prostate growth, hirsutism and acne. Several have recently been identified with beneficial effects on bone mass [45-47], and provide a new alternative to androgen replacement therapy.

III. CELLULAR BIOLOGY OF THE AR IN THE SKELETON

Because there remains confusion interpreting the skeletal actions of sex steroids as previously noted, the specific mechanisms by which androgens affect skeletal homeostasis are becoming the focus of intensified research [1, 48]. As a classic steroid hormone, the biological cellular signaling responses to androgen are mediated through the AR, a ligand-inducible transcription factor. ARs have been identified in a variety of cells found in bone [49]. Characterization of AR expression in these cells thus clearly identifies bone as a target tissue for androgen action. The direct effects of androgen that influence the complex processes of

proliferation, differentiation, mineralization and gene expression in the osteoblast are being characterized, but much remains to be established. Androgen effects on bone may also be indirectly modulated and/or mediated by other autocrine and paracrine factors in the bone microenvironment. The rest of this chapter will review recent progress on the characterization of androgen action in bone.

A. Molecular Mechanisms of Androgen Action in Bone Cells: The AR

Direct characterization of AR expression in a variety of tissues, including bone, was made possible by the cloning of the AR cDNA [50, 51]. The AR is a member of the class I (so-called classical or steroid) nuclear receptor superfamily, as are the ERα and ERß isoforms, the progesterone receptor, the mineralocorticoid and glucocorticoid receptor [52]. Steroid receptors are transcription factors with a highly-conserved modular design characterized by three functional domains: the transactivation, DNA binding and ligand binding domains. In the absence of ligand, the AR protein is generally localized in the cytoplasmic compartment of target cells in a large complex of molecular chaperones, consisting of loosely bound heat-shock, cyclophilin and other accessory proteins [53]. Interestingly, in the unliganded form, AR conformation is unique with a relatively unstructured amino-terminal transactivation domain [54]. As lipids, androgens can freely diffuse through the plasma membrane to bind the AR to induce a conformational change. Once bound by ligand, the AR dissociates from the multiprotein complex, translocates to the nucleus and recruits coactivators or corepressors that demonstrate expression that can be cell-type specific [55], allowing the formation of homodimers (or potentially heterodimers) that activate a cascade of events in the nucleus [56]. Bound to DNA, the AR influences transcription and/or translation of a specific network of genes, leading to the specific cellular response to the steroid.

A steroid hormone target tissue is frequently defined as one that possesses the steroid receptor, at a functional level, with a measurable response in the presence of hormone. Bone

tissue clearly meets this standard with respect to androgen. Colvard *et al.* first reported the presence of AR mRNA and specific androgen binding sites in normal human osteoblastic cells [57]. The abundance of both AR and ER proteins was similar, suggesting that androgens and estrogens each play important roles in skeletal physiology (Fig. 2). Subsequent reports have confirmed AR mRNA expression and/or the presence of androgen binding sites in both normal and clonal, transformed osteoblastic cells derived from a variety of species [16, 58-62]. The size of the AR mRNA transcript in osteoblasts (about 10 kb) is similar to that described in prostate and other tissues [50], as is the size of the AR protein analyzed by Western blotting (~110 kDa) [16]. There are reports of two isoforms of AR protein in human osteoblast-like cells (~110 and ~97 kDa) [63] as first described in human prostatic tissue [64]. It appears these isoforms do not possess similar functional activities in bone, particularly with respect to effects on proliferation [65]. The number of specific androgen binding sites in osteoblasts varies, depending on methodology and the cell source, from 1,000-14,000 sites/cell [16, 61, 63, 66], but is in a range seen in other androgen target tissues. Furthermore, the binding affinity of the AR found in osteoblastic cells ($K_d = 0.5$-2×10^{-9}) is typical of that found in other tissues. Androgen binding is specific, without significant competition by estrogen, progesterone or dexamethasone [16, 57, 63]. Finally, testosterone and DHT appear to have relatively similar binding affinities [16, 58]. All these data are consistent with the notion that the direct biologic effects of androgenic steroids in osteoblasts are mediated at least in part via classic mechanisms associated with the AR as a member of the steroid hormone receptor superfamily described above.

-- Insert Figure 2 here --

In addition to the classical AR present in bone cells, several other androgen-dependent signaling pathways have been described. Specific binding sites for weaker adrenal androgens (such as DHEA) have been described [67]; DHEA can also transactivate AR [9], thus raising the possibility that DHEA or similar androgenic compounds may also have direct effects in bone.

DHEA and its metabolites may also bind and activate additional receptors, including ER, peroxisome proliferator activated receptor-α and pregnane X receptor [68]. Bodine *et al.* [69] showed that DHEA caused a rapid inhibition of c-*fos* expression in human osteoblastic cells that was more robust than seen with the classical androgens (DHT, testosterone, androstenedione). In addition, DHEA may inhibit bone resorption by osteoclasts when in the presence of osteoblasts, likely through changes in osteoprotgerin (OPG) and receptor activator of NFκB ligand (RANKL) concentrations [70]. Alternatively, androgens may be specifically bound in osteoblastic cells by a novel 63-kDa cytosolic protein [71]. In addition, there are reports of distinct AR polymorphisms identified in different races that may have biological impact on androgen responses [72], but to date none have an effect with respect to bone tissue [73]. These different isoforms have the potential to interact in distinct fashions with other signaling molecules, such as c-Jun [74]. Finally, androgens may regulate osteoblast activity via rapid nongenomic mechanisms [75, 76] through membrane receptors displayed at the bone cell surface [77]. The role and biologic significance of these non-classical signaling pathways in androgen-mediated responses in bone remains controversial, and most data suggests that genomic signaling may be the more significant regulator in bone and other tissues [78-81].

B. Localization of AR Expression in Osteoblastic Populations

Ultimately, bone mass is determined by two biological processes: formation and resorption. Distinct cell types mediate these processes. The bone-forming cell, the osteoblast, synthesizes bone matrix, regulates mineralization and is responsive to most calciotropic hormones. The osteoclast is responsible for bone resorption. Clues about the potential sequella of AR signaling might be derived from a better understanding of the cell-types in which expression is documented. *In vivo* analysis has demonstrated significant expression of AR in all cells of the osteoblast lineage including osteoblasts, osteocytes and osteoclasts [82]. Interestingly, ARs are also expressed in bone marrow stromal [83] and mesenchymal precursor

11

cells [84], pluripotent cells that can differentiate into muscle, bone and fat. Androgen action

may modulate precursor differentiation toward the osteoblast and/or myoblast lineage, while

inhibiting differentiation toward the adipocyte lineage [85]. These effects on stromal

differentiation could underlie some of the well-described consequences of androgen

administration on body composition including increased muscle mass [86]. To date, it has not

been established how significant the contribution is, of the increased muscle mass associated

with androgen administration, to positively influence bone quality.

In the bone microenvironment, the localization of AR expression has been described in

intact human bone by Abu *et al* using immunocytochemical methods [49]. In developing bone

from young adults, ARs were predominantly expressed in active osteoblasts at sites of bone

formation (Fig. 3). ARs were also observed in osteocytes embedded in the bone matrix.

Importantly, both the pattern of AR distribution and the level of expression were similar in males

and in females. Furthermore, AR is observed in bone marrow and stromal/osteoblast precursor

cells [83]. In addition, expression of the AR has been characterized in cultured osteoblastic cell

populations isolated from bone biopsy specimens, determined at both the mRNA level and by

binding analysis [63]. Expression varied according to the skeletal site of origin and age of the

donor of the cultured osteoblastic cells: AR expression was higher at cortical and

intramembranous bone sites, and lower in trabecular bone. This distribution pattern correlates

with androgen-responsiveness in the bone compartment. AR expression was highest in

osteoblastic cultures generated from young adults, and somewhat lower in samples from either

prepubertal or senescent bone. Data indicate preferential nuclear staining of AR in males at

sexual maturity, suggesting activation and translocation of the receptor in bone when

androgenic steroid levels are elevated, consistent with androgen regulation of AR levels [87,

88]. Again, no differences were found between male and female samples, suggesting that

differences in receptor number *per se* do not underlie development of a sexually dimorphic

skeleton. Since androgens are so important in bone development at the time of puberty, it is not

surprising that ARs are also present in epiphyseal chondrocytes [49, 89]. The expression of

ARs in such a wide variety of cell types known to be important for bone modeling during

development, and remodeling in the adult, provides evidence for direct actions of androgens in

bone and cartilage tissue. These results illustrate the complexity of androgen effects on bone.

Although bone is a target tissue with respect to androgen action, the mechanisms and cell types

by which androgens exert their effects on bone biology remain incompletely characterized. An

additional complexity in terms of mechanism is that androgens may influence bone directly by

activation of the AR, or indirectly after aromatization of androgens into estrogens with subsequent

activation of ER, as described above.

-- Insert Figure 3 here --

C. Regulation of AR Expression

The regulation of AR expression in osteoblasts is incompletely understood. Homologous

regulation of AR mRNA by androgen has been described that is tissue specific; up-regulation by

androgen exposure is seen in a variety of mesenchymal cells including osteoblasts [60, 62, 87,

88] whereas in prostate and smooth muscle tissue, down-regulation is observed after androgen

exposure [87, 90] (Fig. 4). The androgen mediated up-regulation observed in osteoblasts

occurs, at least in part, through changes in AR gene transcription [87, 88]. No effect, or even

inhibition, of AR mRNA by androgen exposure in other osteoblastic models has also been

described [63, 91]. Interestingly, a novel property of the AR is that binding of androgen

increases AR protein levels, that has been shown in osteoblastic cells as well [88]. This

property distinguishes AR from most other steroid receptor molecules that are down-regulated

by ligand binding. The elevated AR protein levels may be a consequence of increased stability

mediated by androgen binding, resulting from N-terminal and C-terminal interactions [92], but

the stability of AR protein in osteoblastic cells has not been determined to date. The

mechanism(s) that underlie tissue specificity in autologous AR regulation, and the possible

biological significance of distinct autologous regulation of AR, is not yet understood. It is possible that AR up-regulation by androgen in bone may result in an enhancement of androgen responsiveness at times when androgen levels are rising or elevated.

-- Insert Figure 4 here --

Quantitative determination of the level of receptor expression during osteoblast differentiation is difficult to achieve in bone slices. However, analysis of AR, ERα and ERß mRNA and protein expression during osteoblast differentiation *in vitro* demonstrates that each receptor displays differentiation-stage distinct patterns in osteoblasts (Fig. 5) [93]. The levels of AR expression increase throughout osteoblast differentiation with the highest AR levels seen in mature osteoblast/osteocytic cultures. These results suggest that an important compartment for androgen action may be mature, mineralizing osteoblasts, and indicate that osteoblast differentiation and steroid receptor regulation are intimately associated. Given that the osteocyte is the most abundant cell type in bone, and a likely mediator of focal bone deposition and response to mechanical strain [94], it is not surprising that androgens may also augment the osteoanabolic effects of mechanical strain in osteoblasts [95].

-- Insert Figure 5 here --

AR expression in osteoblasts can be up-regulated by exposure to other steroid hormones, including glucocorticoids, estrogen or 1,25-dihydroxyvitamin D_3 [63]. Whether additional hormones, growth factors or agents influence AR expression in bone is not known. Further, whether the AR in osteoblasts undergoes post-translational processing that might influence receptor signaling (stabilization, phosphorylation, *etc.*) as described in other tissues [96, 97], and the potential functional implications [98, 99], are also unknown. Ligand-independent activation of AR has also been described in other tissues [100], but has not been explored in bone.

Steroid receptor transcriptional activity, including that of the AR, is strongly influenced by transcriptional regulators such as coactivators or corepressors [101, 102]. These

coactivators/corepressors can influence the downstream signaling of nuclear receptors; their levels are influenced by the cellular context, and these coregualtors can differentially affect specific promoters. AR specific coactivators have been identified [103], many of which interact with the ligand binding domain of the receptor [104]. Expression and regulation of these modulators may thus influence the ability of steroid receptors to regulate gene expression in bone [105], but this remains underexplored with respect to androgen action. The specific coactivator/corepressor profile present in cells representing different bone compartments (*i.e.*, periosteal cells, proliferating or mineralizing cells) may help determine the activity of the selective receptor modulators such as SARMS described above.

IV. THE CONSEQUENCES OF ANDROGEN ACTION IN BONE CELLS

A. Effects of Androgens on Proliferation and Apoptosis

Evidence suggests that androgens act directly on the osteoblast and there are reports, some in clonal osteoblastic cell lines, of modulatory effects of gonadal androgen treatment on proliferation, differentiation, matrix production and on mineral accumulation [106]. Not surprisingly, androgen has been shown to influence bone cells in a complex fashion. As an example, the effect of androgen on osteoblast proliferation has been shown to be biphasic in nature, with enhancement following short or transient treatment but significant inhibition following longer treatment. As a case in point, Kasperk *et al* [107, 108] demonstrated in osteoblast-like cells in primary culture (murine, passaged human) that a variety of androgens in serum-free medium increase DNA synthesis ([^{3}H]thymidine incorporation) and cell counts. Testosterone and nonaromatizable androgens (DHT and fluoxymesterone) were nearly equally effective regulators. Yet the same group [109] reported that prolonged DHT treatment inhibited normal human osteoblastic cell proliferation (cell counts) in cultures pretreated with DHT. In addition, Benz *et al.* have shown that prolonged androgen exposure in the presence of serum inhibited proliferation (cell counts) by 15-25% in a transformed human osteoblastic line (TE-85) [58].

Testosterone and DHT again were nearly equally effective regulators. Hofbauer *et al* [110] examined the effect of DHT exposure on proliferation in hFOB/AR-6, an immortalized human osteoblastic cell line stably transfected with an AR expression construct (with ~4,000 receptors/cell). In this line, DHT treatment inhibited cell proliferation by 20-35%. Consistent with stimulation, Somjen *et al* have demonstrated increased creatine kinase specific activity in male osteoblastic cells after exposure to DHT for 24 hours [111]. Although these various studies employed different model systems (transformed osteoblastic cells vs. second to fourth passage normal human cells) and culture conditions (including differences in the state of osteoblast differentiation, receptor number, phenol red-containing vs. phenol red-free, or serum containing vs. serum-free), it appears exposure time is an important variable. Clear time dependence for the response to androgen has been shown by Wiren *et al* [112], where osteoblast proliferation was stimulated at early treatment times, but with more prolonged DHT treatment osteoblast viability decreased (Fig. 6). This result was AR dependent (inhibitable by coincubation with flutamide), and was observed in both normal rat calvarial osteoblasts and in AR stably transfected MC-3T3 cells. In mechanistic terms, reduced viability was associated with overall reduction in mitogen-activated (MAP) kinase signaling and with inhibition of *elk-1* gene expression, protein abundance and extent of phosphorylation. The inhibition of MAP kinase activity after chronic androgen treatment again contrasts with stimulation of MAP kinase signaling and AP-1 transactivation observed with brief androgen exposure [112], that may be mediated through non-genomic mechanisms [75, 113, 114].

-- Insert Figure 6 here --

As a component of control of osteoblast survival, it is also important to consider the process of programmed cell death, or apoptosis [115]. In particular, as the osteoblast population differentiates *in vitro*, the mature bone cell phenotype undergoes apoptosis [116]. With respect to the effects of androgen exposure, chronic DHT treatment has been shown to result in enhanced osteoblast apoptosis in both proliferating osteoblastic (at day 5) and in mature osteocytic

cultures (day 29) [117]. In this report, inhibition observed with DHT treatment was opposite to

inhibitory effects on apoptosis seen with E_2 treatment (Fig. 7). An androgen-mediated increase

in the Bax/Bcl-2 ratio was also observed, predominantly through inhibition of Bcl-2 and was

dependent on functional AR. Overexpression of *bcl-2* or RNAi knockdown of *bax* abrogated the

effects of DHT, indicating that increased Bax/Bcl-2 was necessary and sufficient for androgen-

enhanced apoptosis. The increase in the Bax/Bcl-2 ratio was at least in part a consequence of

reductions in Bcl-2 phosphorylation and protein stability, consistent with inhibition of MAP

kinase pathway activation after DHT treatment as noted above. *In vivo* analysis of calvaria in

AR-transgenic male mice demonstrated enhanced TUNEL staining in both osteoblasts and

osteocytes, and was observed even in areas of new bone growth [117]. This may not be

surprising, given an association between new bone growth and apoptosis [118], as has been

observed in other remodeling tissues and/or associated with development and tissue

homeostasis [119]. Apoptotic cell death could thus be important in making room for new bone

formation and matrix deposition, which may have clinical significance by influencing bone

homeostasis and bone mineral density [120]. Thus, mounting evidence suggests that chronic

androgen treatment does not increase osteoblast number or viability in the mature bone

compartment. It may also be that the inhibitory action of androgens in osteoblasts, especially in

the endosteal compartment, are important for the relative maintenance of cortical width (which is

similar between males and females) given the strong stimulation at the periosteal surface, such

that the skeleton does not become excessively large and heavy during development.

-- Insert Figure 7 here --

B. Effects of Androgens on Differentiation of Osteoblastic Cells

Osteoblast differentiation can be characterized by changes in alkaline phosphatase activity

and/or alterations in the expression of important extracellular matrix proteins, such as type I

collagen, osteocalcin and osteonectin. Effects of androgens on expression of these marker

activities/proteins are poorly described and inconsistent. For example, enhanced osteoblast differentiation, as measured by increased matrix production, has been shown to result from androgen exposure in both normal osteoblasts and transformed clonal human osteoblastic cells (TE-89). Androgen treatment appeared to increase the proportion of cells expressing alkaline phosphatase activity, thus representing a shift toward a more differentiated phenotype [107]. Kasperk *et al*. subsequently reported dose-dependent increases in alkaline phosphatase activity in both high and low-alkaline phosphatase subclones of SaOS2 cells [121], and human osteoblastic cells [109]. However, there are also reports, in a variety of model systems, of androgens either inhibiting [110] or having no effect on alkaline phosphatase activity [62, 122], which may reflect both the complexity and dynamics of osteoblastic differentiation. Androgen-mediated increases in type I α-1 collagen protein and mRNA levels [58, 121, 122], and increased osteocalcin secretion [109], have also been described. Consistent with increased collagen production, androgen treatment has also been shown to stimulate mineral accumulation in a time and dose-dependent manner [62, 109, 123]. However, transgenic mice with targeted overexpression of AR in the osteoblast lineage showed decreased levels of most bone markers *in vivo* in total RNA extracts derived from long bone samples, including decreased collagen, osterix and osteocalcin gene expression [28]. These results suggest that, under certain conditions, androgens may enhance osteoblast differentiation and could thus play an important role in the regulation of bone matrix production and/or organization. On the other hand, many positive anabolic effects of androgen may be limited to distinct osteoblastic populations, for example in the periosteal compartment [1, 28].

C. Direct Effects of Androgens on Other Cell Types in the Skeleton

Potential modulation of osteoclast action by androgen is suggested by reports of AR expression in the osteoclast [82]. Androgen treatment reduces bone resorption of isolated osteoclasts [124], inhibits osteoclast formation [125] and that stimulated by parathyroid hormone

(PTH) [126], and may play a direct role regulating aspects of osteoclast activity in AR null mice [127]. Indirect effects of androgen to modulate osteoclasts via osteoblasts are indicated by the increase in OPG levels following testosterone treatment in osteoblasts [128] and in skeletally-targeted AR-transgenic male mice [28]. In addition, DHEA treatment has been shown to decrease in the OPG/RANKL ratio in osteoblastic cells and inhibit osteoclast activity in coculture [70]. Androgen may be a less significant determinant of bone resorption *in vivo* than estrogen [129, 130], although this remains controversial [131].

As with effects noted in osteoblastic populations, androgens regulate chondrocyte proliferation and expression. Although some of the consequences of androgen action are mediated after metabolic conversion to estrogen, which limits long bone growth, nonaromatizable androgen stimulates longitudinal bone growth [132]. AR expression has been demonstrated in cartilage [133], and androgen exposure promotes chondrogenesis as shown with increased creatine kinase and DNA synthesis after androgen exposure in cultured epiphyseal chondrocytes [89, 134]. Increased [^{35}S]sulfate incorporation into newly synthesized cartilage [135] and the increased alkaline phosphatase activity [136] are androgen mediated. Regulation of these effects are obviously complex, as they were dependent on the age of the animals and the site from which chondrocytes were derived. Thus, in addition to effects on osteoblasts, multiple cell types in the skeletal milieu are regulated by androgen exposure.

D. Interaction with Other Factors to Modulate Bone Activity

The effects of androgens on osteoblast activity must certainly also be considered in the context of the very complex endocrine, paracrine and autocrine milieu in the bone microenvironment. Systemic and/or local factors can act in concert, or can antagonize, to influence bone cell function. This has been well described with regard to modulation of the effects of estrogen on bone [see for example 137, 138, 139]. Androgens have also been shown to regulate well-known modulators of osteoblast proliferation or function. The most extensively

characterized growth factor influenced by androgen exposure is transforming growth factor-ß (TGF-ß). TGF-ß is stored in bone (the largest reservoir for TGF-ß) in a latent form, and has been show to be a mitogen for osteoblasts [140, 141]. Androgen treatment has been shown to increase TGF-ß activity in human osteoblast primary cultures. The expression of some TGF-ß mRNA transcripts (apparently TGF-ß2) was increased, but no effect on TGF-ß1 mRNA abundance was observed [69, 108] but also see [142]. At the protein level, specific immunoprecipitation analysis reveals DHT mediated increases in TGF-ß activity to be predominantly TGF-ß2 [69, 109]. DHT has also been shown to inhibit both TGF-ß gene expression and TGF-ß-induced early gene expression that correlates with growth inhibition in this cell line [110]. The TGF-ß-induced early gene has been shown to be a transcription factor that may mediate some TGF-ß effects [143]. These results are consistent with the notion that TGF-ß may mediate androgen effects on osteoblast proliferation. On the other hand, TGF-ß1 mRNA levels are increased by androgen treatment in human clonal osteoblastic cells (TE-89), under conditions where osteoblast proliferation is slowed [58]. Thus, the specific TGF-ß isoform may determine osteoblast responses. It is interesting to note that *in vivo*, orchiectomy (ORX) drastically reduces bone content of TGF-ß levels, and testosterone replacement prevents this reduction [144]. These data support the findings that androgens influence cellular expression of TGF-ß, and suggest that the bone loss associated with castration is related to a reduction in growth factor abundance induced by androgen deficiency.

Other growth factor systems may also be influenced by androgens. Conditioned media from DHT treated normal osteoblast cultures are mitogenic, and DHT pretreatment increases the mitogenic response to fibroblast growth factor and to insulin like growth factor II (IGF-II) [108]. In part, this may be due to slight increases in IGF-II binding in DHT treated cells [108], as IGF-I and IGF-II levels in osteoblast conditioned media are not affected by androgen [108, 145]. Although most studies have not found regulation of IGF-I or IGF-II abundance by androgen exposure [16, 108, 145], there is a report that IGF-I mRNA levels are significantly up-regulated by

DHT [146]. Androgens may also modulate expression of components of the AP-1 transcription

factor [69] or AP-1 transcriptional activation [112]. Thus, androgens may modulate osteoblast

differentiation via a mechanism whereby growth factors or other mediators of differentiation are

regulated by androgen exposure.

Finally, androgens may modulate responses to other important osteotropic

hormones/regulators. Testosterone and DHT specifically inhibit the cAMP response elicited by

PTH or parathyroid hormone-related protein (PTHrP) in the human clonal osteoblast-like cell

line SaOS-2 while the inactive or weakly active androgen 17α-epitestosterone had no effect.

This inhibition may be mediated via an effect on the PTH receptor-G_s-adenylyl cyclase [147-

149]. The production of prostaglandin E_2 (PGE$_2$), another important regulator of bone

metabolism, is also affected by androgens. Pilbeam and Raisz showed that androgens (both

DHT and testosterone) were potent inhibitors of both parathyroid hormone and interleukin-1

stimulated PGE$_2$ production in cultured neonatal mouse calvaria [150]. The effects of androgens

on parathyroid hormone action and PGE$_2$ production suggest that androgens could act to

modulate (reduce) bone turnover in response to these agents.

Finally, both androgen [151] and estrogen [138, 152] [but see 153] inhibit production of

interleukin-6 by osteoblastic cells. In stromal cells of the bone marrow, androgens have been

shown to have potent inhibitory effects on the production of interleukin-6 and the subsequent

stimulation of osteoclastogenesis by marrow osteoclast precursors [154]. Interestingly, adrenal

androgens (androstenediol, androstenedione, DHEA) have similar inhibitory activities on

interleukin-6 gene expression and protein production by stromal cells [154]. The loss of

inhibition of interleukin-6 production by androgen may also contribute to the marked increase in

bone remodeling and resorption that follows ORX, in addition to modulation of osteoclast activity

through changes in the OPG/RANKL ratio as noted above. Moreover, androgens inhibit the

expression of the genes encoding the two subunits of the IL-6 receptor (gp80 and gp130) in the

murine bone marrow, another mechanism which may blunt the effects of this osteoclastogenic

cytokine in intact animals [155]. In these aspects, the effects of androgens seem to be very similar to those of estrogen, which may also inhibit osteoclastogenesis via mechanisms that involve interleukin-6 inhibition and/or OPG/RANKL ratio changes.

V. THE SKELETAL EFFECTS OF ANDROGEN: ANIMAL STUDIES

The effects of androgens on bone remodeling have been examined fairly extensively in animal models. Much of this work has been in species not perfectly suited to reflect human bone metabolism (rodents), and certainly the field remains incompletely explored. Nevertheless, animal models do provide valuable insights into the effects of androgens at organ and cellular levels. Many of the studies of androgen action have been performed in male rats, in which rapid skeletal growth occurs until about 4 months of age, at which time epiphyseal growth slows markedly (although never completely ceases at some sites). Recently, many studies have also employed mice as genetic models. Because the effects of androgen deficiency may be different in growing and more mature animals [156], it is appropriate to consider the two situations independently.

A. Effects on Epiphyseal Function and Bone Growth during Skeletal Development and Puberty

In most mammals there is a marked gender difference in bone morphology. The mechanisms responsible for these differences are complex, and presumably involve both androgenic and estrogenic actions. Estrogens are particularly important for the regulation of epiphyseal function, and act to reduce the rate of longitudinal growth via influences on chondrocyte proliferation and action, as well as on the timing of epiphyseal closure [157]. Androgens appear to have opposite effects, and tend to promote long bone growth, chondrocyte maturation and metaphyseal ossification. Androgen deficiency retards those processes [158].

Nevertheless, excess concentrations of androgen will accelerate aging of the growth plate and reduces growth potential [159], possibly via conversion to estrogens.

Although the specific roles of sex steroids in the regulation of epiphyseal growth and maturation remain somewhat unresolved, there is evidence that androgens do have direct effects independent of those of estrogen. For instance, testosterone injected directly into the growth plates of rats increases plate width [160]. In a model of endochondral bone development based on the subcutaneous implantation of demineralized bone matrix in castrate rats, both testosterone and DHT increase the incorporation of calcium during osteoid formation [123]. Interestingly, in this model androgens reduced the incorporation of [^{35}S]sulfate into glycosaminoglycans early in the developing cartilage. In sum, these data support the contention that androgens play a direct role in chondrocyte physiology, but how these actions are integrated with those of other regulators is unclear.

Finally, during childhood and adolescence, skeletal development is characterized by marked expansion of cortical proportions and increasing trabecular density. During this process, the skeleton develops distinctly in males and females, most significantly at the periosteal surface. Thus, sex differences in skeletal morphology and physiology occur at or around puberty. For that reason, it is hypothesized that gender differences, particularly with respect to "bone quality" and architecture, i.e. predominantly bone width, are modulated by the sex steroids estrogen and androgen. Consistent with this, a distinct response to estrogen and androgen has been described *in vivo* especially in cortical bone. At the periosteum, estrogen suppresses while androgen stimulates new bone formation, yet conversely at the endosteal surface estrogen stimulates but androgen strongly suppresses formation [see 28]. Again these two sex steroids may act in opposition in some situations at distinct bone compartments. Thus, estrogen decreases but androgen increases radial growth in cortical bone through periosteal apposition. These distinct responses to estrogen and androgen during growth likely play an important role in determining sexual dimorphism of the skeleton, i.e. that male bones are wider

23

but not thicker than females [161].   Young men do have larger bone areas than women with

increased whole bone cross-sectional area, particularly at peripheral sites [162].   Interestingly,

low levels of estrogen (in the obligate presence of androgen) may also be important for

stimulation of periosteal bone formation during development [37].   Androgens are also essential

for the production of peak total-body bone mass in males [163].   Finally, androgens are known

to interact with the growth hormone - IGF system in the coordination of skeletal growth.   Growth

hormone deficiency in males has no net effect on endosteal growth but reduced by half

expansion at the periosteal surface [164], underscoring the co-dependence of these two

hormonal systems in the control of pubertal skeletal change.

-- Insert Figure 8 here –

B. Mature Male Animals

Results from animal studies also support an effect of androgen on bone formation in the

mature animal.   Experimental strategies such as surgical or pharmacological intervention, and

examination of genetic models have all been employed to characterize androgen signaling in

the adult.   In mature rats, castration eventually results in osteopenia and both cortical and

trabecular compartments are affected.   At a time when longitudinal growth has slowed

markedly, pronounced differences as a consequence of castration appear in cortical bone ash

weight per unit length, cross-sectional area, cortical thickness, and bone mineral density (Fig. 8)

[165-168].   Castration results in changes in both trabecular and cortical bone compartments and

dramatic bone loss in trabecular bone is noted in both males and females, but sex-specific

responses are most dimorphic in cortical bone.   For example, distinct effects of androgen are

seen with gonadectomy when comparing the effects of ORX in male *vs.* ovariectomy (OVX) in

female rats.   In Turner's classic study [3], OVX and the associated loss of sex steroids in the female

generally results in decreased trabecular area with increased osteoclast number.   In cortical bone,

an increase at the periosteal surface is seen with circumferential enlargement (Fig. 9A), but a

decrease in endosteal labeling in females. In sum, these results demonstrate that estrogen protects trabecular bone predominantly through inhibition of osteoclast activity/recruitment, but has an inhibitory action at the periosteal surface as noted above [for example, see 169]. In the male, ORX with the attendant loss of sex steroids also results in decreased trabecular area with increased osteoclast number. But in contrast with the female, periosteal formation in cortical bone is reduced with the loss of androgen (Fig. 9B). Androgen treatment is effective in suppressing the acceleration of bone remodeling normally seen after ORX [170]. This divergent trend in the periosteal response to castration in male and female animals abolishes the sexual dimorphism usually present in radial bone growth. In the intact animal, the stimulation of endosteal formation by estrogen compensates for the lack of periosteal formation, thus leading to no difference in cortical width or biomechanical strength between the sexes. Nevertheless, factors that influence periosteal apposition may constitute an important therapeutic class, since periosteal bone formation is often a neglected determinant of bone strength [161]. ORX shows either little net effect [164] or slight reductions on the endosteal surface in males, likely due to increased resorption. Consistent with this, increased intracortical resorption cavities are reported to result from ORX [165, 171]. As might be expected in light of these changes, breaking strength (N) can be decreased in cortical bone [164]. In addition, it appears that ORX affects cranial development more than OVX [172], suggesting that androgen action is particularly important in intramembraneous bone.

-- Insert Figure 9 here --

In addition to changes in bone size at the periosteal surface, trabecular bone volume is reduced rapidly after castration as well [165, 173], and osteopenia becomes pronounced with time [48]. It is likely this bone loss appears to result from increased bone resorption, as it is associated with increased resorption cavities, osteoclasts, and blood flow [165, 166]. Dynamic histomorphometric and biochemical measures of bone remodeling increase quickly after ORX [173, 174], with evidence of increased osteoclast numbers only 1 week after castration [173].

These changes include an increase in osteoblastic activity as well as increased bone resorption, reflecting an initial high turnover state that is followed by a reduction in remodeling rates and osteopenia. In the SAMP6 mouse, a model of accelerated senescence in which osteoblastic function is impaired, the rise in remodeling following ORX is blunted, which has been interpreted as evidence that the early changes after gonadectomy are dependent on osteoblast-derived signals [175]. As noted above, androgens reduce osteoclast formation and activity [125], which may be partially mediated by increased OPG levels [28, 128]. The initial phase of increased bone remodeling activity subsides with time [166, 174] and by 4 months there is evidence of a depression in bone turnover rates in some skeletal areas (Fig. 10) [166]. As in younger animals, indices of mineral metabolism are not altered by these changes in skeletal metabolism [168]. Careful histomorphometric analysis of androgen action in ORX male mice by Ohlsson and workers has shown that the bone sparing effect of AR activation in trabecular bone is distinct from the bone-sparing effect of ERα at that site [176]. The analysis demonstrated that AR activation does preserve the number of trabeculae, but does not preserve thickness or volumetric density, nor mechanical strength in cortical bone.

-- Insert Figure 10 here --

As a potential model for the effects of hypogonadism in humans [see 48], animal models therefore suggest an early phase of high bone turnover and bone loss after ORX, followed by a reduction in remodeling rates and osteopenia. The remodeling imbalance responsible for loss of bone mass appears complex, as there are changes in rates of both bone formation and resorption, and patterns that vary from one skeletal compartment to another. These overall changes may be similar to those noted in female animals after castration, in which a loss of estrogen signaling has been associated with an early stimulation of osteoblast progenitor differentiation, an even greater increase in osteoclast numbers, with bone resorption and bone loss [177].

D. Androgens in the Female Animal

Of course androgens are present in females as well as males and may affect bone metabolism. In castrate female rats, DHT administration suppresses elevated concentrations of bone resorption markers, as well the increase in osteocalcin levels [178]. However, alkaline phosphatase activity increases further. Additional evidence to support the contention that androgens play a role in females includes the fact that antiandrogens are capable of evoking osteopenia in intact (*i.e.,* fully estrogenized) female rats [4, 179]. This result suggests that androgens can provide crucial support to bone mass independent of estrogens in females. Of interest, the character of the bone loss induced by flutamide suggested that estrogen prevents bone resorption, whereas androgens stimulate bone formation. In periosteal bone, DHT and testosterone appear to stimulate bone formation after ORX in young male rats, whereas in castrate females they suppress bone formation [3], perhaps reflecting an interaction or synergism between sex steroids and their effects on bone. There is also some information concerning androgen action in females in additional animal models, including primates. For instance, in adult female cynomolgus monkeys, testosterone treatment increased cortical and trabecular bone density as well as biomechanical strength [180]. As noted above, although post-menopausal women can be effectively treated with androgens, combination therapy with estrogen and androgen is more beneficial than either steroid alone [6-8]. This result has recently been confirmed in an animal model [181].

E. Gender specificity

In most mammals, there is a marked gender difference in morphology that results in a sexually dimorphic skeleton. The mechanisms responsible for these differences are necessarily complex, and presumably involve both androgenic and estrogenic actions on the skeleton. It is becoming increasingly clear that estrogens are particularly important for the regulation of epiphyseal function and act to reduce the rate of longitudinal growth via influences on

chondrocyte proliferation and function, as well as on the timing of epiphyseal closure [157]. Androgens, on the other hand, appear to have many opposite effects to estrogen on the skeleton. Androgens tend to promote long bone growth, chondrocyte maturation, and metaphyseal ossification as noted above. Furthermore, the most dramatic effect of androgens is on bone size, in particular cortical thickness [180], as androgens appear to have gender-specific effects on periosteal bone formation to inhibit or stimulate growth [3]. This difference of course has important biomechanical implications, with thicker bones being stronger bones [161]. Furthermore, the response of the adult skeleton (to the same intervention) results is distinct responses in males and females. For example, in a model of disuse osteopenia, antiorthostatic suspension results in significant reduction in bone formation rate at the endosteal perimeter in males. In females however, a decrease in bone formation rate occurred along the periosteal perimeter [182]. Gender-specific responses *in vivo* and *in vitro* [for example, see 111], and the mechanism(s) that underlie such responses in bone cells, may thus have significant implications in treatment options for metabolic bone disease.

VI. ANIMAL MODELS OF ALTERED ANDROGEN RESPONSIVENESS

The specific contribution of AR signaling *in vivo* has also been approached using genetic animal models with global AR modulation, including the testicular feminization (Tfm) model of AIS [169, 183] and with (non-targeted) global AR knockout mice [127, 184]. The Tfm (AR deficient) male rat provides an interesting model for the study of the unique effects of androgens in bone. In these Tfm rats, androgens are presumed to be incapable of action, but estrogen and androstenedione concentrations are considerably higher than in normal males [185, 186]. Clear increases also exist in Tfm male rats in serum concentrations of calcium, phosphorus, and osteocalcin, whereas IGF-1 concentrations are decreased. Estimates of bone mass suggest that Tfm rats have reduced longitudinal and radial growth rates, but that trabecular volume and density are similar to those of normal rats. In selected sites, measures of bone mass and

remodeling were intermediate between normal male and female values. However, castration reduced bone volume markedly in Tfm male rats, suggesting a major role for estrogens as well in skeletal homeostasis (Fig. 11). This model again indicates that androgens have an independent role to play in normal bone growth and metabolism, but the model is complex and not easily dissected. Meticulous analysis in Tfm mice by Vanderschueren *et al* [169] has also shown that the positive effects of testosterone on cortical bone are generally mediated by stimulation of periosteal bone formation, which was absent in Tfm mice. Histomorphometric analysis shows that AR-mediated testosterone action is essential for periosteal bone formation (in male mice), and also contributes to trabecular bone maintenance. This is very similar to the study of humans with the androgen insensitivity syndrome. Marcus *et al.* [42] reported that there is a deficit in bone mineral density in women with androgen insensitivity even when compliance with estrogen replacement is excellent. However, inadequate estrogen replacement appeared to worsen the deficit, and other environmental factors are difficult to quantitate. Thus, in Tfm models, ORX demonstrates the importance of AR in mediating the positive effects of androgen to contribute to trabecular bone maintenance, and in cortical bone particularly at the periosteal surface [169, 183].

-- Insert Figure 11 here --

The bone phenotype that develops in a global AR null (ARKO) male mouse model is a high-turnover osteopenia, with reduced trabecular bone volume and a significant stimulatory effect on osteoclast function [127, 184, 187]. As expected, bone loss with ORX in male ARKO mice was only partially prevented by treatment with aromatizable testosterone due to the lack of AR.

A final model for AR modulation is represented by overexpression of AR in AR-transgenic mice [28], constructed with full-length AR under the control of the 3.6 kb type I collagen promoter, with AR overexpression in osteoblast stromal precursors and throughout the osteoblast lineage. AR-transgenic mice are the only model with skeletally-targeted manipulation of AR expression, and demonstrate enhanced sensitivity to androgen without changes in

circulating steroids or androgen administration [28]. AR overexpression in this model results in a complex phenotype predominantly in males, with increased trabecular bone mass (with increased trabecular number but not thickness) in the setting of inhibition of resorption due to reduced osteoclast activity. In addition, cortical formation is altered with periosteal expansion but inhibition of inner endosteal deposition (Fig. 12), consistent with the known effects of androgen to stimulate periosteal apposition and opposite to effects of estrogen on these compartments. Inhibition of osteoclastic resorption may be responsible for altered trabecular morphology, consistent with reduced osteoclast activity and increased trabecular bone volume observed with androgen therapy in rodents and humans. The dramatic inhibition of bone formation at the endosteal envelope may underlie the modest decrease in cortical bone area and subsequent reductions in biomechanical properties observed. Notably, the bone phenotype observed in AR-transgenic mice is consistent with many of the known effects of androgen treatment on the skeleton. Combined, studies employing genetic models indicate that AR expressed in bone can be a direct mediator of androgen action to influence skeletal development and homeostasis.

-- Insert Figure 9 here --

VII. EFFECTS ON THE PERIOSTEUM: THE ROLE OF AR VS. AROMATIZATION OF TESTOSTERONE

As noted above, androgen-mediated AR transactivation is likely a key determinant of the sexually-dimorphic pattern of periosteal apposition that is most clearly demonstrated in male AR-transgenic mice in the absence of hormone administration [28]. Furthermore, essentially all of the alterations induced by ORX (in both growing and mature animals) can be prevented at least in part by replacement with either testosterone or nonaromatizable androgens [3, 171, 188-192]. These results strongly suggest that aromatization of androgens to estrogens cannot fully explain the actions of androgens on bone metabolism.

However estrogens also seem play a role in the effects of androgen on periosteal

apposition. Although AR activity is essential, low levels of estrogens are likely required for

optimal stimulation of periosteal growth [193], as observed in aromatase deficiency even in

males [37]. Estrogens may also help prevent bone loss following castration in male animals.

Vanderschueren *et al.* [168] reported that estradiol (and nandrolone) was capable of not only

preventing the increase in biochemical indices stimulated by ORX, but also preventing cortical

and trabecular bone loss. In fact, estradiol resulted in an absolute increase in trabecular bone

volume not achieved with androgen replacement. Similarly, estrogen was reported to

antagonize the increase in blood flow resulting from castration and to increase bone ash weight

more consistently than testosterone. Although data thus far available are far from complete,

these studies raise obvious questions of the overlap between the actions of androgens and

estrogens in bone and/or the consequences of skeletal adaptation to changes in bone

morphology.

The gender reversal of estrogen replacement in male animals is also instructive.

Nonaromatizable androgens are capable of preventing or reversing osteopenia and abnor-

malities in bone remodeling in OVX females [3, 194]. These actions apparently result from the

suppression of trabecular bone resorption as well as stimulation of periosteal bone formation

[194]. Very similar results have been reported following the treatment of OVX animals with

DHEA [3]. Moreover, blockage of androgen action with an AR antagonist in female rats already

treated with an estrogen antagonist increases bone loss and indices of osteoclast activity more

than treatment with an estrogen antagonist alone [195], again indicating that ovarian androgens

(apart from estrogens) exert a protective effect on bone in females. Analogously,

androstenedione reduces (although does not abrogate) trabecular bone loss and remodeling

alterations in OVX animals treated with an aromatase inhibitor [196, 197]. This protective effect

was blocked by the addition of an AR antagonist [196]. Finally, whereas aromatase inhibition in

male rats reduces bone mass, the large increase in remodeling induced by ORX does not occur

in these animals [38]. Also, ORX in ERKO mice further reduces bone mass [80]. The latter

observation implicates a role for androgens in the maintenance of bone mass in ERKO mice.

VIII. SUMMARY

The effects of androgens on bone health are obviously both pervasive and complex.

Androgens are important in the maintenance of a healthy skeleton, and have been shown to

stimulate bone formation in the periosteum. Androgens influence skeletal modeling and

remolding by multiple mechanisms through effects on osteoblasts, osteoclasts and even

perhaps an influence on the differentiation of pluripotent stem cells toward distinct lineages.

The specific effects of androgen on bone cells are mediated directly through an AR-signaling

pathway, but there are also indirect contributions to overall skeletal health through aromatization

and ER signaling. The effects of androgens are particularly dramatic during growth in boys, but

almost certainly play an important role during this period in girls as well. Throughout the rest of

life, androgens affect skeletal function in both sexes. Still poorly characterized, more needs to

be done to unravel the mechanisms by which androgens influence the physiology and

pathophysiology of bone, and there remains much to be learned about the roles of androgens at

all levels. The interaction of androgens and estrogens, and how their respective actions can be

utilized for specific diagnostic and therapeutic benefit, are important but unanswered issues.

With an increase in the understanding of the nature of androgen effects will come greater

opportunities to use their positive actions in the prevention and treatment of a wide variety of

skeletal disorders.

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Figure Legends.

Figure 1. Principle conversions and major enzyme activities involved in androgen synthesis and metabolism. Steroid hormone synthesis involves metabolism of cholesterol, with dehydrogenation of pregnenolone producing progesterone that can serve as a precursor for the other gonadal steroid hormones. DHEA, dehydroepiandrosterone; CYP11A, cytochrome P450 cholesterol side chain cleavage enzyme; CYP17, cytochrome P450 17α hydroxylase/17,20 lyase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; CYP19, aromatase cytochrome P450.

Figure 2. Nuclear androgen and estrogen receptor binding in normal human osteoblast-like cells. Dots represent the mean calculated number of molecules per cell nucleus for each cell strain. (*Left*) Specific nuclear binding of [^{3}H]R1881 (methyltrienolone, an androgen analog) in 12 strains from normal men and 13 strains from normal women. (*Right*) Specific nuclear [^{3}H]estradiol binding in 15 strains from men and 15 strains from women. The horizontal lines indicate the mean receptor concentrations. Adapted from Colvard *et al* [57] and used with permission.

Figure 3. The localization of AR in normal tibial growth plate and adult osteophytic human bone. a) Morphologically, sections of the growth plate consist of areas of endochondral ossification with undifferentiated (*small arrow head*), proliferating (*large arrow heads*), mature (*small arrow*) and hypertrophic (*large arrow*) chondrocytes. Bar = 80 µm. An inset of an area of the primary spongiosa is shown in b. b) Numerous osteoblasts (*small arrow heads*) and multinucleated osteoclasts (*large arrow heads*) on the bone surface. Mononuclear cells within the bone marrow are also present (*arrows*). Bar = 60 µm. c) In the growth plate, AR is predominantly expressed by hypertrophic chondrocytes (*large arrow heads*). Minimal expression is observed in the mature chondrocytes (*small arrow heads*). The receptors are rarely observed in the proliferating chondrocytes (*arrow*). d) In the primary spongiosa, the AR

is predominantly and highly expressed by osteoblasts at modeling sites (*arrow heads*). Bar = 20 μm. e) In the osteophytes, AR is also observed at sites of endochondral ossification in undifferentiated (*small arrow heads*), proliferating (*large arrow heads*), mature (*small arrows*), and hypertrophic-like (*large arrow*) chondrocytes. Bar = 80 μm. f) A higher magnification of e) showing proliferating, mature, and hypertrophic-like chondrocytes (*large arrows, small arrows, and very large arrows* respectively) Bar = 40 μm. g) At sites of bone remodeling, the receptors are highly expressed in the osteoblasts (*small arrow heads*) and also in mononuclear cells in the bone marrow (*large arrow heads*). Bar = 40 μm. h) AR is not detected in osteoclasts (*small arrow heads*) Bar = 40 μm. B, Bone: C, Cartilage; BM, Bone marrow. Adapted from Abu *et al* [49] and used with permission.

Figure 4. Dichotomous regulation of AR mRNA levels in osteoblast-like and prostatic carcinoma cell lines after exposure to androgen (A) Time course of changes in AR mRNA abundance after DHT exposure in human SaOS-2 osteoblastic cells and human LNCaP prostatic carcinoma cells. To determine the effect of androgen exposure on hAR mRNA abundance, confluent cultures of either osteoblast-like cells (SaOS-S) or prostatic carcinoma cells (LNCaP) were treated with 10^{-8} M DHT for 0, 24, 48, or 72 h. Total RNA was then isolated and subjected to RNase protection analysis with 50 μg total cellular RNA from SaOS-2 osteoblastic cells and 10 μg total RNA from LNCaP cultures. (B) Densitometic analysis of AR mRNA steady-state levels. The AR mRNA to ß-actin ratio is expressed as the mean ± SEM compared to the control value from three to five independent assessments. Adapted from Wiren *et al* [87] and used with permission.

Figure 5. Expression analyses of ERα, ERß and AR during *in vitro* differentiation in normal rat osteoblastic (rOB) cultures. (A) Normal rOB cells were cultured for the indicated number of days during proliferation, matrix maturation, mineralization and postmineralization stages. Total RNA

was isolated and subjected to relative RT-PCR analysis using primers specific for rat ERα, ERß and AR or rat GAPDH. Reverse transcription was conducted with PCR carried out for 40 cycles for the steroid receptors, with parallel reactions performed using GAPDH primers for 25 cycles (all in the linear range. Bands for rat ERα at the predicted 240 bp, rat ERß at 262 bp, rat AR at 276 bp and GAPDH at 609 bp are shown. (B) Analyses of ERα, ERß and AR mRNA relative abundance. Semi-quantitative analysis of mRNA steady-state expression by relative RT-PCR was performed after scanning the negative image of the photographed gels. Data are expressed in arbitrary units as the ratio of receptor abundance to GAPDH expression, then normalized to expression values at day 4 in pre-confluent cultures. Data represent mean ± SEM. Adapted from Wiren *et al* [93] and used with permission.

Figure 6. Complex effect of androgen on DNA accumulation in osteoblastic cultures. (A) Kinetics of DHT response in proliferating colAR-MC3T3 cultures measured with colorimetric (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay. Cultures of stably transfected colAR-MC3T3 continuously with 10^{-8} M DHT for 2 days led to increased MTT accumulation, but longer treatment for 3 or 5 days resulted in inhibition. Data are mean ± SEM of six to eight dishes with six wells/dish. *$p < 0.05$; **$p < 0.01$ (vs control). Adapted from Wiren *et al* [112] and used with permission.

Figure 7. Characterization of osteoblast apoptosis: results of androgen and estrogen treatment during proliferation (day 5) and during differentiation into mature osteoblast/osteocytes cultures (day 29). Apoptosis was assessed at day 5 or day 29 after continuous DHT and E_2 treatment (both at 10^{-8} M). Apoptosis was induced by etoposide treatment in proliferating cultures and by serum starvation for 48 h in confluent cultures before isolation, replaced with 0.1% BSA. (A) Analysis of apoptosis after evaluating DNA fragmentation by cytoplasmic nucleosome enrichment at day 5. The data are expressed as mean ± SEM (n=6) from two independent

experiments. **$p < 0.01$, ***$p < 0.001$ (vs. control).   (B) Analysis of apoptosis by cytoplasmic

nucleosome enrichment analysis at day 29. The data are expressed as mean ± SEM (n=6)

from two independent experiments. **$p < 0.01$ vs. control.   Adapted from Wiren *et al* [112]and

used with permission.

 Figure 8. Microphotographs of 200 μm thick mid-diaphyseal cross sections from 24-month old

(a) intact and (b) ORX rats taken in a polarization microscope. Magnification X 14.

Adapted from Danielson *et al* [166] and used with permission.

Figure 9. A. The effect of ovariectomy (OVX) on periosteal bone formation rate. The mean ±

SEM (vertical bar) and tetracycline labeling period (horizontal line) for intact controls (-○-) and

OVX (-●-) rats are shown as a function of time after OVX. $p < 0.01$ for all OVX time points

compared to intact controls. B. The effect of ORX on periosteal bone formation rate. The mean

± SEM and tetracycline labeling period for intact controls (-▲-) and ORX (- △ -) are shown as a

function of time after ORX. $p < 0.01$ for all ORX time points compared to same labeling period

in intact controls. Adapted from Turner *et al* [Turner, 1990 #4] and used with permission.

Figure 10. Evolution of the bone calcium turnover rate after castration (ratio of castrated/sham-

operated animals). *$p < 0.05$. Adapted from Verhas *et al* [165] and used with permission.

Figure 11. Cancellous bone volume (BV/TV in %) of the proximal metaphysis of the tibia in

male, female, Tfm, and orchiectomized male rats. Adapted from Vanderschueren *et al* [185]

and used with permission.

Figure 12. Characterization of cortical bone formation in AR-transgenic (AR-tg) mice. Dynamic

histomorphometric analysis was performed in cortical bone after fluorescent imaging

microscopy in AR-tg males (n=6-8). Mineralizing surface as a percent of bone surface (MS/BS), mineral apposition rate (MAR), bone formation rate (BFR) at both the endosteal and periosteal surfaces were determined in wildtype (wt) and AR-tg mice. *$p < 0.05$. Adapted from Wiren *et al* [28] and used with permission.

Abbreviations:

5α-androstane-3α or 3β,17β-diol (3α/β-androstanediol)

androgen receptor (AR)

AR null (ARKO)

bone formation rate (BFR)

dehydroepiandrosterone (DHEA)

5α-dihydrotestosterone, (DHT)

estradiol (E2)

estriol (E3)

estrogen receptor-α (ERα)

estrone (E1)

17ß-hydroxysteroid dehydrogenase (17ß-HSD)

insulin like growth factor (IGF)

mineral apposition rate (MAR)

mineralizing surface as a percent of bone surface (MS/BS)

mitogen-activated kinase (MAP)

osteoprotegerin (OPG)

orchiectomy (ORX)

ovariectomy (OVX)

prostaglandin E_2 (PGE_2)

parathyroid hormone (PTH)

parathyroid hormone-related protein (PTHrP)

receptor activator of nuclear factor kappa B (NF-kB) ligand (RANKL)

selective AR modulators (SARMs)

transforming growth factor-ß (TGF-ß)

testicular feminization (Tfm)

Androgens Receptor Expression and Steroid Action in Bone

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INTRODUCTION

Much of the research describing the general action of gonadal steroids in bone has focused on the specific effects of estrogen (see Chapter 14) because of the obvious impact of the menopause on skeletal health. Nevertheless, it is clear that androgens, in both men and women, also have important beneficial effects on skeletal development and on the maintenance of bone mass. It has been demonstrated that androgens (a) influence growth plate maturation and closure, helping to determine longitudinal bone growth during development, (b) mediate regulation of cortical bone mass in a fashion distinct from estrogen, leading to a sexually dimorphic skeleton, (c) modulate peak bone mass acquisition, and (d) influence trabecular (cancellous) bone and inhibit bone loss (Wiren, 2005). In castrated animals, replacement with nonaromatizable androgens (e.g., 5α-dihydrotestosterone, DHT) yields beneficial effects that are clearly distinct from those observed with estrogen replacement (Turner *et al.,* 1989; Turner *et al.,* 1990b). In intact females, blockade of the androgen receptor (AR) with the specific AR antagonist hydroxyflutamide results in osteopenia (Goulding and Gold, 1993). Furthermore, treatment with nonaromatizable androgen alone in females results in improvements in bone mineral density (Coxam *et al.,* 1996). Finally, combination therapy with estrogen and androgen in postmenopausal women is more beneficial than either steroid alone (Castelo-Branco *et al.,* 2000; Miller *et al.,* 2000; Raisz *et al.,* 1996), indicating nonparallel pathways of action. Taken together, these reports illustrate the divergent actions of androgens and estrogens on the skeleton. Thus, in both men and women it is probable that androgens and estrogens each have important yet distinct functions during bone development, and in the subsequent maintenance of skeletal homeostasis in the adult. With an increased awareness of the importance of the effects of androgen on skeletal homeostasis (Vanderschueren *et al.,* 2004), and the potential to make

use of this information for the treatment of bone disorders, much nevertheless remains to be learned.

THE ROLE OF ANDROGEN METABOLISM

Metabolism of Androgens in Bone: Aromatase, 17β-hydroxysteroid Dehydrogenase (17β-HSD), and 5α-Reductase Activities

Sex steroids, ultimately derived from cholesterol, are synthesized predominantly in gonadal tissue, the adrenal gland and placenta as a consequence of enzymatic conversions. The major pathways are shown in Figure 1. After peripheral metabolism, androgenic activity is represented in a variety of steroid molecules that include testosterone. There is accumulating evidence that in a range of tissues the eventual cellular effects of testosterone may not be the result (or not only the result) of direct action of testosterone, but may also reflect the effects of sex steroid metabolites formed as a consequence of local enzyme activities. The most important testosterone metabolites likely to influence bone are 5α-DHT (the result of 5α reduction of testosterone) and estradiol (formed by the aromatization of testosterone). Testosterone and DHT are the major and most potent androgens, with androstenedione (the major circulating androgen in women) and dehydroepiandrosterone (DHEA) as immediate androgen precursors that exhibit weak androgen activity (Mo *et al.,* 2006). DHEA, although a weaker androgen, is still considered anabolic (Labrie *et al.,* 2006). In men, the most abundant circulating androgen metabolite is testosterone, while concentrations of other weaker androgens like androstenedione and DHEA-sulfate are similar between males and females. Downstream metabolites of DHT and androstenedione are inactive at the AR, and include 5α-androstane-3α or 3β,17β-diol (3α/β-androstanediol) and 5α-androstanedione. Data suggest

Peripheral androgen metabolism
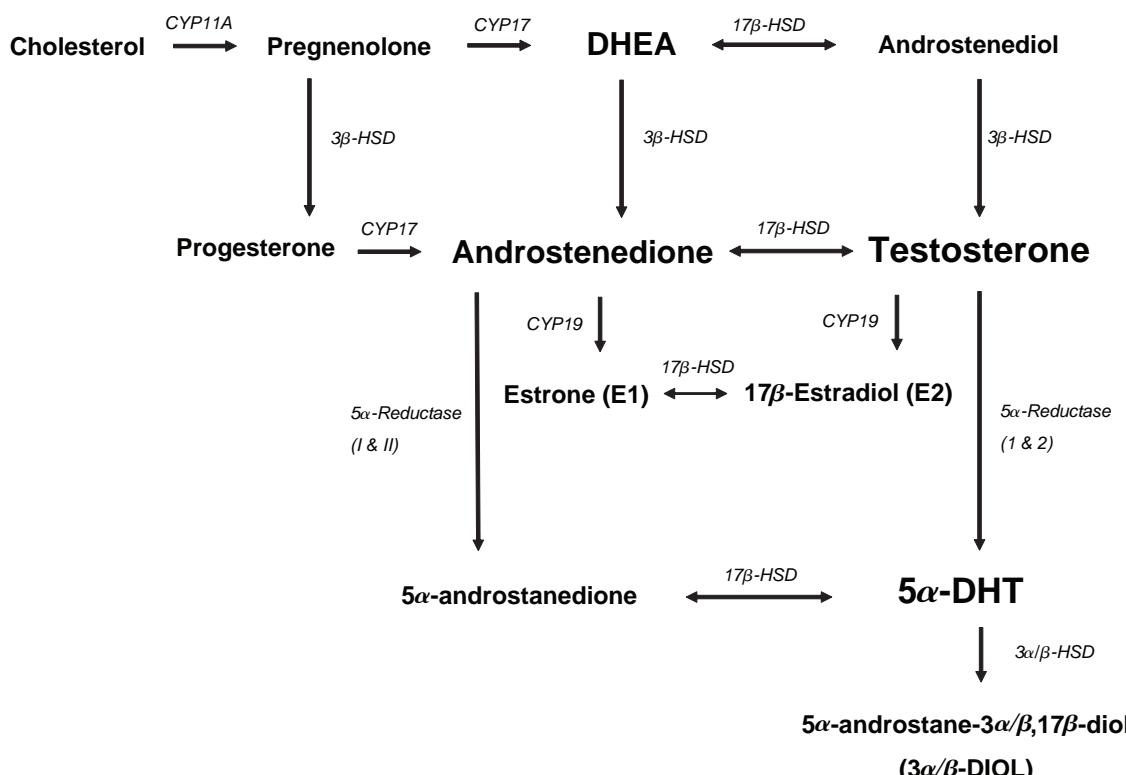
FIGURE 1 Principle conversions and major enzyme activities involved in androgen synthesis and metabolism. Steroid hormone synthesis involves metabolism of cholesterol, with dehydrogenation of pregnenolone producing progesterone that can serve as a precursor for the other gonadal steroid hormones. DHEA, dehydroepiandrosterone; CYP11A, cytochrome P450 cholesterol side chain cleavage enzyme; CYP17, cytochrome P450 17α hydroxylase/17,20 lyase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; CYP19, aromatase cytochrome P450.

that aromatase cytochrome P450 (the product of the CYP19 gene), 17β-hydroxysteroid dehydrogenase (17β-HSD), and 5α-reductase activities are all present in bone tissue as described later, at least to some measurable extent in some compartments, but the biologic relevance of each remains somewhat controversial.

An important enzymatic arm of testosterone metabolism involves the biosynthesis of estrogens from androgen precursors, catalyzed by aromatase. Aromatase is well known to be expressed and regulated in a pronounced tissue-specific manner (Simpson *et al.,* 1994), and also exhibits species differences, given the low levels in mice. Modest levels of aromatase activity have been reported in bone from mixed cell populations derived from both sexes (Nawata *et al.,* 1995; Sasano *et al.,* 1997; Schweikert *et al.,* 1995) and from osteoblastic cell lines (Nakano *et al.,* 1994; Purohit *et al.,* 1992; Tanaka *et al.,* 1993). Aromatase expression in intact bone has also been documented by *in situ* hybridization and immunohistochemical analysis (Sasano *et al.,* 1997). At least in vertebral bone, the mesenchymal distal promoter I.4 is predominantly utilized (Shozu and Simpson, 1998). The enzyme kinetics in bone

cells seem to be similar to those in other tissues, although the V_{max} may be increased by glucocorticoids (Tanaka *et al.,* 1993). Aromatase mRNA is expressed predominantly in lining cells, chondrocytes, and some adipocytes; however, there is little to no detectable expression in osteoclasts, or in cortical bone in mice (Wiren *et al.,* 2004b). Thus, whether the level of aromatase activity in bone is high enough to produce physiologically relevant concentrations of steroids locally remains an open question. Nevertheless, in the male only 15% of circulating estrogen is produced in the testes, with the remaining 85% produced by peripheral metabolism that could theoretically include bone as one site of conversion (Gennari *et al.,* 2004).

Aromatase catalyzes the metabolism of adrenal and testicular C19 androgens (androstenedione and testosterone) to C18 estrogens (estrone and estradiol), thus producing the potent estrogen estradiol (E2) from testosterone, and the weaker estrogen estrone (E1) from its adrenal precursors androstenedione and DHEA (Nawata *et al.,* 1995). Thus, because of aromatase activity, systemically administered testosterone may have effects either at the AR (with either testosterone or DHT as ligands) or at the estrogen

receptor (ERα or ERβ). Typically in the circulation, E2 will make up to 40% of total estrogen, E1 will make up an additional 40%, with estriol (E3) comprising the remaining 20% of total estrogen (Lin *et al.,* 2006). In addition to aromatase itself, osteoblasts contain enzymes that are able to interconvert estradiol and estrone (17β-HSD, see following section), and to hydrolyze estrone sulfate, the most abundant estrogen in the circulation, to estrone (steroid sulfatase) (Muir *et al.,* 2004; Purohit *et al.,* 1992). Dexamethasone and 1α,25(OH)$_2$D$_3$ synergistically enhance aromatase activity and aromatase mRNA expression in human osteoblast-like cells (Nawata *et al.,* 1995). In addition, both leptin and 1α,25(OH)$_2$D$_3$ treatment increased aromatase activity in human mesenchymal stem cells during osteogenesis, but not during adipogenesis (Pino *et al.,* 2006). Additional studies are needed to better define expression, given the potential importance of the enzyme, and its regulation by a variety of mechanisms (including androgens and estrogens) in other tissues (Abdelgadir *et al.,* 1994; Simpson *et al.,* 1994).

The clinical impact of aromatase activity, with an indication of the importance of conversion of circulating androgen into estrogen, is shown in reports of women and men with aromatase deficiencies who present with a skeletal phenotype (Jones *et al.,* 2007). The presentation of men with aromatase deficiency is very similar to that of a man with ERα deficiency (Smith *et al.,* 1994), namely an obvious delay in bone age, lack of epiphyseal closure, and tall stature with high bone turnover and osteopenia (Gennari *et al.,* 2004). These findings suggest that aromatase (and likely estrogen action) has an important role to play during skeletal development in the male. Interestingly, natural mutation is remarkably rare with only seven males and seven females reported to date (Jones *et al.,* 2007). In addition, estrogen therapy of males with aromatase deficiency has been associated with an increase in bone mass (Gennari *et al.,* 2004) particularly noted in the growing skeleton (Bouillon *et al.,* 2004). Inhibition of aromatization pharmacologically with nonsteroidal inhibitors (such as vorozole or letrozole) results in modest decreases in bone mineral density and changes in skeletal modeling in young growing orchidectomized males (Vanderschueren *et al.,* 1997), and less dramatically so in boys with constitutional delay of puberty treated for one year (Wickman *et al.,* 2003), suggesting short-term treatment during growth has limited negative consequences in males. Inhibition of aromatization in older orchidectomized males resembles castration with similar increases in bone resorption and bone loss, suggesting that aromatase activity likely does play a role in skeletal maintenance in males (Vanderschueren *et al.,* 1996). Combined, these studies herald the importance of aromatase activity (and estrogen) in the mediation of some androgen action in bone in both males and females. The characterization of these enzyme activities in bone clearly raises the difficult issue of the origin of androgenic effects

in the skeleton; do they arise solely from direct androgen effects (as is suggested by the actions of nonaromatizable androgens such as DHT) or also from the local or other-site production of estrogenic intermediates? The results described here would indicate that both steroids appear to be important to both male and female skeletal health.

The 17β-HSDs (most of which are dehydrogenase-reductases, except type 5, which is an aldo-keto reductase) have been shown to catalyze either the last step of sex steroid synthesis or the first step of their degradation. This activity produces weak or potent sex steroids via oxidation or reduction, respectively, and can thus also play a critical role in peripheral steroid metabolism. The oxidative pathway forms 17-ketosteroids while the reductive pathway forms 17β-hydroxysteroids. The enzyme reversibly catalyzes the formation of androstenediol (an estrogen) from DHEA, in addition to the biosynthesis of estradiol from estrone, the synthesis of testosterone from androstenedione, and the production of DHT from 5α-androstanedione all via the reductive activity of 17β-HSD. Of the 13 enzyme isotypes of 17β-HSD activity (Lin *et al.,* 2006), either types 1–4 (Feix *et al.,* 2001) or types 1, 3, and 5 (Miki *et al.,* 2007) have been demonstrated in human osteoblastic cells.

5α-reductase is also an important activity with regard to androgen metabolism, because testosterone is converted to the more potent androgen metabolite DHT via 5α-reductase action (Bruch *et al.,* 1992). 5α-reductase activity was first described in crushed rat mandibular bone (Vittek *et al.,* 1974) with similar findings reported in crushed human spongiosa (Schweikert *et al.,* 1980). Two different 5α-reductase genes encode type 1 and type 2 isozymes in many mammalian species (Russell and Wilson, 1994); mRNAs encoding the type 2 isozyme are more abundant than type 1 mRNAs in most male reproductive tissues, whereas the type 1 predominate in peripheral tissues. Human osteoblastic cells express the type 1 isozyme (Issa *et al.,* 2002). Essentially the same metabolic activities were reported in experiments with human epiphyseal cartilage and chondrocytes (Audi *et al.,* 1984). In general, the K$_m$ values for bone 5α-reductase activity are similar to those in other androgen responsive tissues (Nakano *et al.,* 1994; Schweikert *et al.,* 1980). However, the cellular populations in many of these studies were mixed and hence the specific cell type responsible for the activity is unknown. Interestingly, periosteal cells do not have detectable 5α-reductase activity (Turner *et al.,* 1990a), raising the possibilities that the enzyme may be functional in only selected skeletal compartments, and that testosterone may be the active androgen metabolite at this clinically important site.

From a clinical perspective, the general importance of this enzymatic pathway is uncertain, as patients with 5α-reductase type 2 deficiency have normal bone mineral density (Sobel *et al.,* 2006) and no significant correlation

was observed between enzyme activities and bone volume (Bruch *et al.,* 1992). Mutant null mice lacking both 5α-reductase type 1 (and type 2) have been created (Mahendroo *et al.,* 2001), but the effect on the skeleton has not been analyzed. Analysis of the importance of 5α-reductase activity has also been approached with the use of finasteride, an inhibitor of 5α-reductase activity that is selective for type 2 in humans, but comparably inhibits both type 1 and type 2 in rodents (Finn *et al.,* 2006). Finasteride treatment of male animals does not recapitulate the effects of castration (Rosen *et al.,* 1995). Furthermore, inhibition of type 1 5α-reductase using MK-434 does not block the effect of testosterone to reduce bone turnover in orchidectomized rats (Borst *et al.,* 2005). Combined, these studies suggest that reduction of testosterone to DHT is not the major determinant in the effects of gonadal hormones on bone. Consistent with this finding, testosterone therapy in hypogonadal older men, either when administered alone or when combined with finasteride, increases bone mineral density, again suggesting that DHT is not essential for the beneficial effects of testosterone on bone (Amory *et al.,* 2004). Thus, the available clinical data regarding the importance of 5α-reductase activity remain uncertain, and the impact of this enzyme, which isozyme may be involved, whether it is uniformly present in all cell types involved in bone modeling/remodeling, or whether local activity is important in any bone compartment, remain unresolved issues.

The administration of testosterone can stimulate bone formation and inhibit bone resorption, likely through multiple mechanisms that involve both androgen and estrogen receptor-mediated processes. However, there is substantial evidence that some, if in fact not most, of the biologic actions of androgens in the skeleton are mediated by AR signaling in bone. Both *in vivo* and *in vitro* systems reveal the effects of the nonaromatizable androgen DHT to be essentially the same as those of testosterone. In addition, blockade of the AR with the receptor antagonist flutamide results in osteopenia as a result of reduced bone formation (Goulding and Gold, 1993). Consistent with this result, complete androgen insensitivity results in a significant decrease in bone mineral density in spine and hip sites (Sobel *et al.,* 2006) even in the setting of strong compliance with estrogen treatment (Marcus *et al.,* 2000). These reports clearly indicate that androgens, independent of estrogenic metabolites, have primary effects on osteoblast function. However, the clinical reports of subjects with aromatase deficiency also highlight the relevance of metabolism of androgen to biopotent estrogens at least in the circulation, to influence development and/or bone mass maintenance. It thus seems likely that further elucidation of the regulation steroid metabolism, and the potential mechanisms by which androgenic and estrogenic effects are coordinated, will have physiological, pathophysiological, and therapeutic implications.

Synthetic Androgens

In addition to the endogenous steroid metabolites highlighted in Figure. 1, there are also a variety of drugs with androgenic activity. These include anabolic steroids, such as nonaromatizable oxandrolone that can bind and activate AR, albeit with lower affinity than testosterone (Kemppainen *et al.,* 1999). In addition, a class of drugs under extensive development, referred to as selective AR modulators (SARMs), demonstrate tissue-specific agonist or antagonist activities with respect to AR transactivation (Omwancha and Brown, 2006). These orally active nonsteroidal nonaromatizable SARMs are being developed to target androgen action in bone, muscle, fat, and to influence libido but to not exacerbate prostate growth, hirsutism, and acne. Several have recently been identified with beneficial effects on bone mass (Allan *et al.,* 2007; Kearbey *et al.,* 2007; Miner *et al.,* 2007). Thus, this class of drug may provide a new alternative to androgen replacement therapy.

ANDROGEN RECEPTOR AND SKELETAL CELLULAR BIOLOGY

Because there remains confusion clearly interpreting the skeletal actions of circulating sex steroids as noted, the specific mechanisms by which androgens affect skeletal homeostasis are becoming the focus of intensified research (Vanderschueren *et al.,* 2004; Wiren, 2005). As a classic steroid hormone, the biological cellular signaling responses to androgen are mediated through the AR, a ligand-inducible transcription factor. ARs have been identified in a variety of cells found in bone (Abu *et al.,* 1997). Characterization of AR expression in these cells thus clearly identifies bone as a target tissue for androgen action. The direct effects of androgen that influence the complex processes of proliferation, differentiation, mineralization, and gene expression in the osteoblast are being characterized, but much remains to be established. Androgen effects on bone may also be indirectly modulated and/or mediated by other autocrine and paracrine factors in the bone microenvironment. The rest of this chapter will review recent progress on the characterization of androgen action through the AR in bone.

Molecular Mechanisms of Androgen Action in Bone Cells: The AR

Direct characterization of AR expression in a variety of tissues, including bone, was made possible by the cloning of the AR cDNA (Chang *et al.,* 1988; Lubahn *et al.,* 1988). The AR is a member of the class I (so-called classical or steroid) nuclear receptor superfamily, as are the ERα and ERβ isoforms, the progesterone receptor, the mineralocorticoid, and glucocorticoid receptor (Mangelsdorf *et al.,*

Model for AR genomic interaction
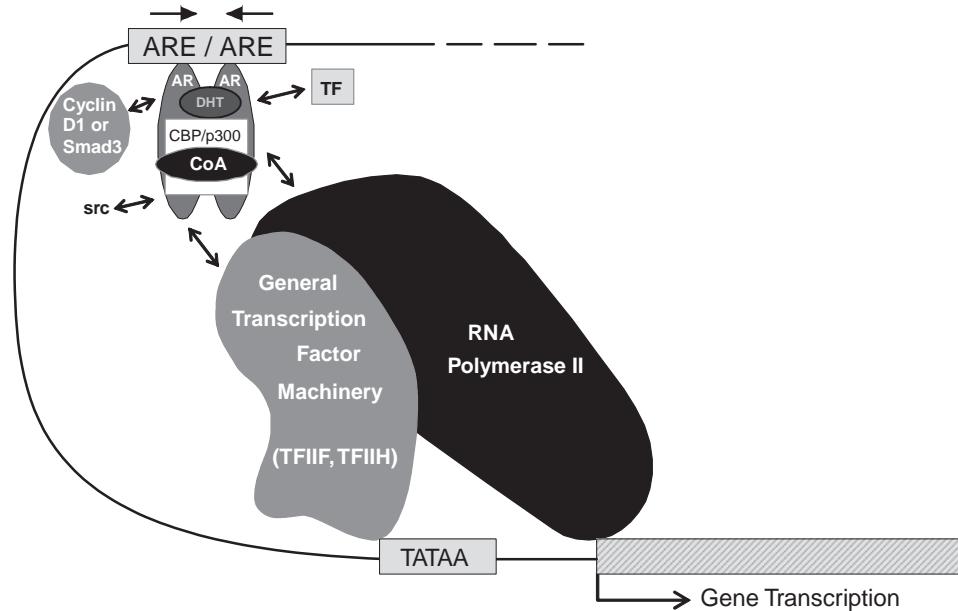
FIGURE 2 Model of AR regulation of gene expression. Binding of androgen promotes high-affinity dimerization, followed by DNA binding at the androgen response element (ARE) in an androgen-responsive gene promoter. Coactivators may remodel/modify chromatin through histone acetylase activity to open chromatin structure (Spencer *et al.*, 1997), or act as a bridge to attract transcription factors (TFs) that target binding of TATA-binding protein to the TATAA sequence (Beato and Sanchez-Pacheco, 1996). Conversely, corepressors act through histone deacetylase activity to reduce accessibility of promoter sequences. Phosphorylation of receptor may result from activation of SRC by growth factors (Kraus *et al.*, 2006). Smad3 can act as either a coactivator or corepressor (Hayes *et al.*, 2001; Kang *et al.*, 2002), while cyclin D1 is a corepressor of AR transactivation (Leader *et al.*, 2006). AR can also directly contact TFIIH and TFIIF (Lee and Chang, 2003) in the general transcription machinery. Such interactions between the AR and the general transcription machinery, leading to stable assembly, results in recruitment of RNA polymerase II and subsequent increased gene transcription. Downregulation of gene expression can also be AR mediated.

1995). Steroid receptors are transcription factors with a highly conserved modular design characterized by three functional domains: the transactivation, DNA binding, and ligand binding domains. In terms of function, the DNA binding domain targets the receptor complex to a specific DNA sequence known as the hormone response element and has high homology among the steroid receptors; the transactivation function mediates transcriptional regulation of gene expression and is localized in both the amino and carboxyterminal of the molecule; the carboxyterminal ligand binding domain mediates not only ligand binding but also receptor dimerization and nuclear translocation, in addition to transcriptional regulation (Mangelsdorf *et al.,* 1995). In the absence of ligand, the AR protein is generally localized in the cytoplasmic compartment of target cells in a large complex of molecular chaperones, consisting of loosely bound heat-shock, cyclophilin, and other accessory proteins (Picard, 2006). Interestingly, in the unliganded form, AR conformation is unique with a relatively unstructured amino-terminal transactivation domain (Shen and Coetzee, 2005). As lipids, androgens can freely diffuse through the plasma membrane to bind the AR to induce a conformational change. Once bound by ligand, the AR

dissociates from the multiprotein complex, translocates to the nucleus allowing the formation of homodimers (or potentially heterodimers), and recruits coregulators (coactivators or corepressors), to initiate a cascade of events in the nucleus that influence transcription (Chang *et al.,* 1995). It may be functionally significant that coactivators or corepressors are expressed in a cell type specific manner (Kumar *et al.,* 2006). Bound to DNA, the AR influences transcription and/or translation of a specific network of genes, leading to the specific cellular response to the steroid (see Fig. 2).

A steroid hormone target tissue is generally defined as one that expresses the steroid receptor, at a functional level, and with a measurable response in the presence of the hormone ligand. Bone tissue clearly meets this standard with respect to androgen. AR mRNA and specific androgen binding sites in normal human osteoblastic cells were first reported by these workers (Colvard *et al.,* 1989). The abundance of both AR and ER proteins was similar, suggesting that androgens and estrogens each play important roles in skeletal physiology. Subsequent reports have confirmed AR mRNA expression and/or the presence of androgen binding sites in both normal and clonal, transformed osteoblastic cells derived from a variety of species (Benz *et al.,*

1991; Liesegang *et al.,* 1994; Nakano *et al.,* 1994; Orwoll *et al.,* 1991; Takeuchi *et al.,* 1994; Zhuang *et al.,* 1992). The size of the AR mRNA transcript in osteoblasts (about 10 kb) is similar to that described in prostate and other tissues (Chang *et al.,* 1988), as is the size of the AR protein analyzed by Western blotting (~110 kDa) (Nakano *et al.,* 1994). There are reports of two isoforms of AR protein in human osteoblast-like cells (~110 and ~97 kDa) (Kasperk *et al.,* 1997a) as first described in human prostatic tissue (Wilson and McPhaul, 1994). It appears these isoforms do not possess similar functional activities in bone, particularly with respect to effects on proliferation (Liegibel *et al.,* 2003). The number of specific androgen binding sites in osteoblasts varies, depending on methodology and the cell source, from 1,000–14,000 sites/cell (Kasperk *et al.,* 1997a; Liesegang *et al.,* 1994; Masuyama *et al.,* 1992; Nakano *et al.,* 1994), but is in a range seen in other androgen target tissues. Furthermore, the binding affinity of the AR found in osteoblastic cells ($K_d = 0.5-2 \times 10^{-9}$) is typical of that found in other tissues. Androgen binding is specific, without significant competition by estrogen, progesterone, or dexamethasone (Colvard *et al.,* 1989; Kasperk *et al.,* 1997a; Nakano *et al.,* 1994). Finally, testosterone and DHT appear to have similar though not identical binding affinities for AR (Benz *et al.,* 1991; Nakano *et al.,* 1994). All these data are consistent with the notion that the direct biologic effects of androgenic steroids in osteoblasts are mediated at least in part via classic mechanisms associated with the AR as a member of the steroid hormone receptor superfamily described earlier.

In addition to the classical AR present in bone cells, several other androgen-dependent signaling pathways have been described. Specific binding sites for weaker adrenal androgens (such as DHEA) have been reported (Meikle *et al.,* 1992), thus raising the possibility that DHEA or similar androgenic compounds have direct effects in bone. DHEA can transactivate AR (Mo *et al.,* 2006), but DHEA and its metabolites may also bind and activate additional receptors, including ER, peroxisome proliferator activated receptor-α and pregnane X receptor (Webb *et al.,* 2006). It has been shown that DHEA rapidly inhibited c-*fos* expression in human osteoblastic cells (Bodine *et al.,* 1995), inhibition that was more robust than seen with the classical androgens (DHT, testosterone, androstenedione). In addition, DHEA may inhibit bone resorption by osteoclasts when in the presence of osteoblasts, likely through changes in osteoprotegerin (OPG) and receptor activator of NFγB ligand (RANKL) concentrations (Wang *et al.,* 2006), that are important mediators of osteoclastogenesis. Androgens may also be specifically bound in osteoblastic cells by a novel 63-kDa cytosolic protein (Wrogemann *et al.,* 1991). Finally, androgens may regulate osteoblast activity via rapid nongenomic mechanisms (Kang *et al.,* 2004; Kousteni *et al.,* 2003; Zagar *et al.,* 2004), through membrane receptors displayed at the bone cell surface

(Lieberherr and Grosse, 1994). Nongenomic signaling generally involves either changes in intracellular calcium levels or rapid activation of kinase signaling cascades. However, the role and biologic significance of these nonclassical signaling pathways *in vivo* remains controversial (Neill, 2006), and most data suggest that genomic signaling through specific receptors may be the more significant regulator in bone and other tissues (Centrella *et al.,* 2004; Hewitt *et al.,* 2006; Sims *et al.,* 2003; van der Eerden *et al.,* 2002a; Windahl *et al.,* 2006).

Lastly, there are reports of AR polyglutamine tract (CAG/CAA) polymorphisms, leading to shorter or longer glutamine tract lengths in the receptor protein, which likely have a biological impact on androgen responses (Pettaway, 1999). Enhanced androgen action is associated with shorter AR CAG repeats and, conversely, hypoandrogenic traits are seen in patients with an elongation of more than 37 CAG repeats (Zitzmann *et al.,* 2005). Most studies have failed to find an effect of such polymorphisms in AR on bone mass (Kenny *et al.,* 2005; Valimaki *et al.,* 2005; Van Pottelbergh *et al.,* 2001). An exception to these findings is a reported association in postmenopausal women with long CAG AR isoforms, where spinal bone mineral density (BMD) was significantly lower compared to those with AR short alleles (Retornaz *et al.,* 2006). Different AR isoforms have the potential to interact in distinct fashions with other signaling molecules such as c-Jun (Grierson *et al.,* 1999), and may also influence variation in serum testosterone levels (Crabbe *et al.,* 2007). Thus, such AR CAG polymorphisms may have a significant influence on bone mass, although data remain underdeveloped.

Localization of AR Expression in Osteoblastic Populations

Ultimately, bone mass is determined by two biological processes: formation and resorption. Distinct cell types mediate these processes. The bone-forming cell – the osteoblast – synthesizes bone matrix, regulates mineralization, and is responsive to most calciotropic hormones. The osteoclast is responsible for bone resorption. Clues about the potential sequela of AR signaling might be derived from a better understanding of the cell types in which expression is documented. *In vivo* analysis has demonstrated expression of AR in all cells of the osteoblast lineage including osteoblasts and osteocytes, and in osteoclasts (van der Eerden *et al.,* 2002b). In the bone microenvironment, the localization of AR expression has been described in intact human bone by Abu *et al.* using immunocytochemical methods (Abu *et al.,* 1997). In developing bone from young adults, ARs were predominantly expressed in active osteoblasts at sites of bone formation and in bone marrow cells. ARs were also observed in osteocytes embedded in the bone matrix. Interestingly, both the pattern of AR distribution

and the level of expression were similar in males and in females. Expression of the AR has also been characterized in cultured osteoblastic cell populations isolated from bone biopsy specimens, determined at both the mRNA level and by binding analysis (Kasperk *et al.,* 1997a). AR levels varied according to the skeletal site of origin and age of the donor of the cultured osteoblastic cells: expression was higher at cortical and intramembranous bone sites, and lower in trabecular bone. This distribution pattern may correlate with androgen responsiveness in various bone compartments. AR expression was highest in osteoblastic cultures generated from young adults, and somewhat lower in samples from either prepubertal or senescent bone. Data indicate preferential nuclear staining of AR in males at sexual maturity, suggesting activation and translocation of the receptor in bone when androgenic steroid levels are elevated, consistent with androgen regulation of AR levels at such times (e.g., Wiren *et al.,* 1999; Wiren *et al.,* 1997). Again, no differences were found between male and female samples, suggesting that differences in receptor number per se do not underlie development of a sexually dimorphic skeleton. Because androgens are so important in bone development at the time of puberty, it is not surprising that ARs are also present in epiphyseal chondrocytes (Abu *et al.,* 1997; Carrascosa *et al.,* 1990). The expression of ARs in such a wide variety of cell types known to be important for bone modeling during development, and remodeling in the adult, provides evidence for direct actions of androgens in bone and cartilage tissue. These results also highlight the complexity of androgen effects on bone. Although bone is a target tissue with respect to androgen action, the mechanisms and cell types by which androgens exert their effects on bone biology remain incompletely characterized. An additional complexity in terms of mechanism is that androgens may influence bone directly by activation of the AR, or indirectly after aromatization of androgens into estrogens with subsequent activation of ER as described earlier.

Regulation of AR Expression

The regulation of AR expression in osteoblasts is incompletely characterized. Homologous regulation of AR mRNA by androgen has been described that is tissue-specific; upregulation by androgen exposure is seen in a variety of mesenchymal cells including osteoblasts (Takeuchi *et al.,* 1994; Wiren *et al.,* 1999; Wiren *et al.,* 1997; Zhuang *et al.,* 1992) whereas downregulation is observed after androgen exposure in prostate and smooth muscle tissue (Lin *et al.,* 1993; Wiren *et al.,* 1997). The androgen mediated upregulation observed in osteoblasts, at least in part, occurs through changes in AR gene transcription (Wiren *et al.,* 1999; Wiren *et al.,* 1997). No effect, or even inhibition, of AR mRNA by androgen exposure in other osteoblastic

models has also been described (Hofbauer *et al.,* 1997; Kasperk *et al.,* 1997a). Interestingly, a novel property of the AR is that binding of androgen increases AR protein levels. This property distinguishes AR from most other steroid receptor molecules that are downregulated by ligand binding. At least in part, the elevated AR protein levels may be a consequence of increased stability mediated by androgen binding results from N-terminal and C-terminal interactions (Langley *et al.,* 1998), but the stability of AR in osteoblastic cells has not been determined to date. The mechanism(s) that underlie tissue specificity in autologous AR regulation, and the possible biological significance of distinct autologous regulation of AR, is not established. It is possible that AR upregulation by androgen in bone may result in an enhancement of androgen responsiveness at times when androgen levels are rising or elevated.

Quantitative determination of the level of receptor expression during osteoblast differentiation is difficult to achieve in bone slices. However, analysis of AR, ERα and ERβ mRNA and protein expression during osteoblast differentiation *in vitro* has been described, revealing that each receptor displays differentiation-stage distinct patterns in osteoblasts (Wiren *et al.,* 2002). In contrast to the ERs, AR expression level increases throughout osteoblast differentiation with the highest AR levels seen in mature osteoblast/osteocytic cultures. These results suggest that an important compartment for androgen action may be mature, mineralizing osteoblasts, and also indicate that osteoblast differentiation and steroid receptor regulation are intimately associated. Given that the osteocyte is the most abundant cell type in bone, and a likely mediator of focal bone deposition and response to mechanical strain (Seeman, 2006), it is not surprising that androgens may also augment the osteoanabolic effects of mechanical strain in osteoblasts (Liegibel *et al.,* 2002).

AR expression in osteoblasts can be upregulated by exposure to other steroid hormones, including glucocorticoids, estrogen or 1,25-dihydroxyvitamin D$_3$ (Kasperk *et al.,* 1997a). Whether additional hormones, growth factors, or agents influence AR expression in bone is not known. Further, whether the AR in osteoblasts undergoes post-translational processing that might influence receptor signaling (stabilization, phosphorylation, etc.) as described in other tissues (Ikonen *et al.,* 1994; Kemppainen *et al.,* 1992), and the potential functional implications (Blok *et al.,* 1996; Wang *et al.,* 1999a), are also unknown. Phosphorylation may be of particular interest in osteoblasts, as it is known to positively or negatively influence receptor interaction with coactivators and corepressors (Weigel and Moore, 2007). Ligand-independent activation of AR has also been described in other tissues (Dehm and Tindall, 2006), but has not been explored in bone.

Steroid receptor transcriptional activity, including that of the AR, is strongly influenced by coactivator or corepressor function (He *et al.,* 2006; Yoon and Wong, 2006).

These coregulators can influence the downstream signaling of nuclear receptors; their levels are influenced by the cellular context, and they can differentially affect specific promoters. AR specific coactivators have been identified (MacLean *et al.,* 1997), many of which interact with the ligand binding domain of the receptor (Yeh and Chang, 1996). Expression and regulation of these modulators may thus influence the ability of steroid receptors to regulate gene expression in bone (Haussler *et al.,* 1997), but this remains underexplored with respect to androgen action. The specific coactivator/corepressor profile present in cells representing different bone compartments (i.e., periosteal cells, proliferating or mineralizing cells) may influence the activity of the selective receptor modulators such as SARMs described earlier.

THE CONSEQUENCES OF ANDROGEN ACTION IN BONE CELLS

Effects of Androgens on Proliferation and Apoptosis

Evidence suggests that androgens act directly on the osteoblast and there are reports, some in clonal osteoblastic cell lines, of modulatory effects of gonadal androgen treatment on proliferation, differentiation, matrix production, and on mineral accumulation (Notelovitz, 2002). Not surprisingly, androgen has been shown to influence bone cells in a complex fashion. As an example, the effect of androgen on osteoblast proliferation has been shown to be biphasic in nature, with enhancement following short or transient treatment but significant inhibition following longer treatment. As a case in point, it was demonstrated (Kasperk *et al.,* 1990; Kasperk *et al.,* 1989) in osteoblast-like cells in primary culture (murine, passaged human) that a variety of androgens in serum-free medium increase DNA synthesis ($[^3H]$thymidine incorporation) and cell counts. Testosterone and nonaromatizable androgens (DHT and fluoxymesterone) were nearly equally effective regulators. Yet the same group (Kasperk *et al.,* 1997a) reported that prolonged DHT treatment inhibited normal human osteoblastic cell proliferation (cell counts) in cultures pretreated with DHT. In addition, prolonged androgen exposure in the presence of serum inhibited proliferation (cell counts) by 15–25% in TE-85, a transformed human osteoblastic line (Benz *et al.,* 1991). Testosterone and DHT again were nearly equally effective regulators. Other workers (Hofbauer *et al.,* 1998) examined the effect of DHT exposure on proliferation in hFOB/AR-6, an immortalized human osteoblastic cell line stably transfected with an AR expression construct (with ~4,000 receptors/cell). In this line, DHT treatment inhibited cell proliferation by 20–35%. Consistent with stimulation of proliferation, Somjen *et al.* have demonstrated after exposure to DHT for 24 hours an increase

in creatine kinase specific activity in male osteoblastic cells (Somjen *et al.,* 2006). Although these various studies employed different model systems (transformed osteoblastic cells vs. second to fourth passage normal human cells) and culture conditions (including differences in the state of osteoblast differentiation, receptor number, phenol red-containing vs. phenol red-free, or serum containing vs. serum-free), it appears exposure time is an important variable. Time dependence for the response to androgen has been shown (Wiren *et al.,* 2004a), where osteoblast proliferation was stimulated at early treatment times, but with more prolonged DHT treatment osteoblast viability decreased. This result was AR dependent (i.e., inhibitable by coincubation with flutamide), and was observed in both normal rat calvarial osteoblasts and in stably transfected AR MC-3T3 cells. In mechanistic terms, reduced viability was associated with overall reduction in mitogen-activated (MAP) kinase signaling, and with downstream inhibition of *elk-1* gene expression, protein abundance, and extent of phosphorylation. The inhibition of MAP kinase activity after chronic androgen treatment again contrasts with stimulation of MAP kinase signaling and AP-1 transactivation observed with brief androgen exposure (Wiren *et al.,* 2004a), which may be mediated through nongenomic mechanisms at least *in vitro*.

As an additional component of control of osteoblast abundance, it is important to consider the process of programmed cell death, or apoptosis (Wyllie *et al.,* 1980). Apoptosis is important generally during development and for homeostasis, but it has also been shown that as the osteoblast population differentiates *in vitro*, the mature bone cell phenotype undergoes apoptosis (Lynch *et al.,* 1998). With respect to the effects of androgen exposure, chronic DHT treatment has been shown to result in enhanced osteoblast apoptosis *in vitro* in both proliferating osteoblastic at day 5, and in mature osteocytic mineralizing cultures at day 29 (Wiren *et al.,* 2006). In the same study, the enhancement observed with DHT treatment was opposite to the inhibitory effects on apoptosis seen with E_2 treatment. An androgen-mediated increase in the Bax/Bcl-2 ratio was also observed, predominantly through inhibition of Bcl-2 that was dependent on functional AR. The increase in the Bax/Bcl-2 ratio was at least in part a consequence of reductions in Bcl-2 phosphorylation and protein stability, consistent with inhibition of MAP kinase pathway activation after DHT treatment as noted earlier. Importantly, a similar response was observed *in vivo* with characterization of apoptosis in calvaria harvested from transgenic mice with overexpression of AR targeted throughout the osteoblast lineage. In male transgenic mice with the normal hormonal milieu (i.e., without systemic administration of androgen), enhanced TUNEL staining is observed in bone in both osteoblasts and osteocytes, even in areas of new bone growth (Wiren *et al.,* 2006). This may not be surprising, given an association between new

bone growth and apoptosis (Palumbo *et al.,* 2003), as has been observed in other remodeling tissues and/or associated with development and tissue homeostasis (Lanz *et al.,* 2003). Apoptotic cell death could thus be important in making room for new bone formation and matrix deposition, which may have clinical significance by influencing bone homeostasis and bone mineral density (Miura *et al.,* 2004). Thus, mounting evidence suggests that chronic androgen treatment increases neither osteoblast number nor viability in the mature bone compartment. It is interesting to speculate that, given strong androgen-mediated stimulation at the periosteal surface, such inhibitory action by androgens in osteoblasts at the endosteum (also see next section) is important for the maintenance of cortical width that is similar between males and females. Such a response would help pattern a skeleton in males that does not become excessively large and heavy during development or in the adult.

Effects of Androgens on Differentiation of Osteoblastic Cells

Osteoblast differentiation can be characterized by changes in alkaline phosphatase activity and/or alterations in the expression of important extracellular matrix proteins, such as type I collagen, osteocalcin, and osteonectin. Again, the effects of androgens on expression of these marker activities/proteins are poorly described and results are inconsistent between a variety of model systems. For example, enhanced osteoblast differentiation, as measured by increased matrix production, has been shown to result from androgen exposure in both normal osteoblasts and transformed clonal human osteoblastic cells (TE-89). Androgen treatment appeared to increase the proportion of cells expressing alkaline phosphatase activity, thus representing a shift toward a more differentiated phenotype (Kasperk *et al.,* 1989). Furthermore, the same group subsequently reported dose-dependent increases in alkaline phosphatase activity in both high and low-alkaline phosphatase subclones of SaOS2 cells (Kasperk *et al.,* 1996), and human osteoblastic cells (Kasperk *et al.,* 1997b). However, there are also reports employing a variety of model systems of androgens either inhibiting (Hofbauer *et al.,* 1998) or having no effect on alkaline phosphatase activity (Gray *et al.,* 1992; Takeuchi *et al.,* 1994). These various responses may reflect both the underlying complexity and dynamics of osteoblastic differentiation. Androgen-mediated increases in type Iα-1 collagen protein and mRNA levels (Benz *et al.,* 1991; Gray *et al.,* 1992; Kasperk *et al.,* 1996), and increased osteocalcin secretion (Kasperk *et al.,* 1997b), have also been described. Consistent with increased collagen production, androgen treatment has also been shown to stimulate mineral accumulation in a time- and dose-dependent manner (Kapur and Reddi, 1989; Kasperk *et al.,*

1997b; Takeuchi *et al.,* 1994). However, transgenic mice with targeted overexpression of AR in the osteoblast lineage showed decreased levels of most bone markers *in vivo* in RNA extracts derived from long bone samples, including decreased collagen, osterix, and osteocalcin gene expression (Wiren *et al.,* 2004b). These results suggest that, under certain conditions, androgens may enhance osteoblast differentiation and could thus play an important role in the regulation of bone matrix production and/or organization. On the other hand, many positive anabolic effects of androgen may be limited to distinct osteoblastic populations, for example in the periosteal compartment (Wiren, 2005; Wiren *et al.,* 2004b).

Androgen Effects on Other Cell Types in the Skeleton

Within the bone compartment, AR is detected in macrophages, megakaryocytes, and endothelial cells (Mantalaris *et al.,* 2001). Interestingly, ARs are also expressed in bone marrow stromal and mesenchymal precursor cells (Gruber *et al.,* 1999; Sinha-Hikim *et al.,* 2004), pluripotent cells that can differentiate into a variety of tissues including muscle, bone, and fat. Androgen action may modulate precursor differentiation toward the osteoblast and/or myoblast lineage, while inhibiting differentiation toward the adipocyte lineage (Singh *et al.,* 2003). These effects on stromal differentiation could underlie some of the well-described consequences of androgen administration on body composition including increased muscle mass (Herbst and Bhasin, 2004). Although it is an intriguing hypothesis to propose that androgen-mediated increases in muscle would indirectly increase bone mass through enhanced mechanical loading, to date it has not been established how significant such a contribution is. However, the time course required for oxandrolone (a synthetic testosterone analog) to positively effect bone mass in severely burned children is delayed relative to effects on lean mass, suggesting that alterations in muscle are indeed important (Bi *et al.,* 2007; Murphy *et al.,* 2004). In addition, androgen influences immune cells and hematopoiesis, and could play an indirect role in mediating effects on bone via immune cells. Although plausible, additional studies are needed to establish the importance of such indirect regulation of other tissues by androgen, to positively influence the skeleton.

Importantly, AR expression has also been detected in the osteoclast (Michael *et al.,* 2005; Mizuno *et al.,* 1994; van der Eerden *et al.,* 2002b), including human osteoclasts (Michael *et al.,* 2005), but see (Abu *et al.,* 1997; Noble *et al.,*1998), indicating modulation of osteoclast action by androgen. Thus, it has been shown that androgen treatment reduces bone resorption of isolated osteoclasts (Pederson *et al.,*1999), and inhibits osteoclast formation (Huber *et al.,* 2001) including that stimulated by parathyroid hormone (PTH) (Chen *et al.,* 2001). That AR may play a direct

role in regulating (inhibiting) osteoclast activity is also suggested by the high turnover osteopenia observed in AR null mice (Kawano *et al.,* 2003). Also of importance, androgens indirectly modulate osteoclastogenesis and osteoclast activity through effects mediated by osteoblasts (Michael *et al.,* 2005), consistent with increased levels of OPG observed following testosterone treatment in cultured osteoblasts (Chen *et al.,* 2004), and in both the serum and in bone derived from skeletally targeted AR3.6-transgenic male mice (Wiren *et al.,* 2004b). Thus, although estrogen also inhibits bone resorption, androgen regulation is distinct compared to estrogen utilizing both direct and indirect pathways (Michael *et al.,* 2005). In addition, DHEA treatment has been shown to increase the OPG/RANKL ratio in osteoblastic cells and inhibit osteoclast activity in coculture (Wang *et al.,* 2006). Androgen may be a less significant determinant of bone resorption *in vivo* than estrogen (Falahati-Nini *et al.,* 2000; Oh *et al.,* 2005), although this remains controversial (Leder *et al.,* 2003).

As with effects noted in osteoblastic populations, androgens also regulate chondrocyte proliferation and expression. Although some of the consequences of androgen action are mediated after metabolic conversion to estrogen, which limits long bone growth, nonaromatizable androgen stimulates longitudinal bone growth (Nilsson *et al.,* 2005). AR expression has been demonstrated in biopsies of proximal tibial growth plate cartilage (Nilsson *et al.,* 2003), and androgen exposure promotes chondrogenesis as shown with increased creatine kinase and DNA synthesis after androgen exposure in cultured epiphyseal chondrocytes (Carrascosa *et al.,* 1990; Somjen *et al.,* 1991). Increased [^{35}S]sulfate incorporation into newly synthesized proteoglycan (Corvol *et al.,* 1987) and increased alkaline phosphatase activity (Schwartz *et al.,* 1994) are androgen mediated. Regulation of these effects is obviously complex, as they were influenced by the age of the animals and the site from which the chondrocytes were derived. Thus, in addition to effects on osteoblasts, multiple cell types in the skeletal milieu are regulated by androgen exposure, and all are likely involved in mediating the effects of androgens on the skeleton.

Interaction with Other Factors to Modulate Bone Activity

The effects of androgens on osteoblast activity must certainly also be considered in the context of the very complex endocrine, paracrine, and autocrine milieu in the bone microenvironment. Systemic and/or local factors can act in concert, or can antagonize, to influence bone cell function. This has been well described with regard to modulation of the effects of estrogen on bone (see for example Horowitz, 1993; Kassem *et al.,* 1996; Kawaguchi *et al.,* 1995).

Androgens have also been shown to regulate well-known modulators of osteoblast proliferation or function. The most extensively characterized growth factor influenced by androgen exposure is transforming growth factor-β (TGF-β). TGF-β is stored in bone (the largest reservoir for TGF-β) in a latent form, and has been shown to be a mitogen for osteoblasts (Centrella *et al.,* 1994; Harris *et al.,* 1994). Androgen treatment has been shown to increase TGF-β activity in human osteoblast primary cultures. The expression of some TGF-β mRNA transcripts (apparently TGF-β2) was increased, but no effect on TGF-β1 mRNA abundance was observed (Bodine *et al.,* 1995; Kasperk *et al.,* 1990) but also see (Wang *et al.,* 1999b). At the protein level, specific immunoprecipitation analysis reveals DHT-mediated increases in TGF-β activity to be predominantly TGF-β2 (Bodine *et al.,* 1995; Kasperk *et al.,* 1997b). DHT has also been shown to inhibit both TGF-β gene expression and TGF-β-induced early gene expression that correlates with growth inhibition in this cell line (Hofbauer *et al.,* 1998). The TGF-β-induced early gene has been shown to be a transcription factor that may mediate some TGF-β effects (Subramaniam *et al.,* 1995). These results are consistent with the notion that TGF-β may mediate androgen effects on osteoblast proliferation. On the other hand, TGF-β1 mRNA levels are increased by androgen treatment in human clonal osteoblastic cells (TE-89), under conditions where osteoblast proliferation is slowed (Benz *et al.,* 1991). Thus, the specific TGF-β isoform may determine osteoblast responses. It is interesting to note that *in vivo*, orchiectomy drastically reduces bone content of TGF-β levels, and testosterone replacement prevents this reduction (Gill *et al.,* 1998). Finally, androgen may modulate the levels of certain SMADs (Miki *et al.,* 2007), transcription factors that mediate TGF-β signaling. These data support the findings that androgens influence TGF-β cellular expression or activity, and suggest that the bone loss associated with castration is related to a reduction in growth factor activity induced by androgen deficiency.

Other growth factor systems may also be influenced by androgens. Conditioned media from DHT-treated normal osteoblast cultures are mitogenic, and DHT pretreatment increases the mitogenic response to fibroblast growth factor and to insulin-like growth factor II (IGF-II) (Kasperk *et al.,* 1990). In part, this may be due to slight increases in IGF-II binding in DHT-treated cells (Kasperk *et al.,* 1990), as IGF-I and IGF-II levels in osteoblast conditioned media are not affected by androgen (Canalis *et al.,* 1991; Kasperk *et al.,* 1990). In contrast to effects of estrogen, most studies have not found regulation of IGF-I or IGF-II abundance by androgen exposure (Canalis *et al.,* 1991; Kasperk *et al.,* 1990; Nakano *et al.,* 1994), but see (Gori *et al.,* 1999). Androgens may also modulate expression of components of the AP-1 transcription factor (Bodine *et al.,* 1995) or AP-1 transcriptional activation (Wiren *et al.,* 2004a). Thus, androgens may modulate osteoblast differentiation via a

mechanism whereby growth factors or other mediators of differentiation are regulated by androgen exposure.

Androgens may also modulate responses to other important osteotropic hormones/regulators. Testosterone and DHT specifically inhibit the cAMP response elicited by PTH or parathyroid hormone-related protein (PTHrP) in the human clonal osteoblast-like cell line SaOS-2 whereas the inactive or weakly active androgen 17α-epitestosterone had no effect. This inhibition may be mediated via an effect on the PTH receptor-G_s-adenylyl cyclase (Fukayama and Tashjian, 1989; Vermeulen, 1991). The production of prostaglandin E_2 (PGE_2), another important regulator of bone metabolism, is also affected by androgens. Androgens (both DHT and testosterone) were shown as potent inhibitors of both parathyroid hormone and interleukin1 stimulated PGE_2 production in cultured neonatal mouse calvaria (Pilbeam and Raisz, 1990). The effects of androgens on parathyroid hormone action and PGE_2 production suggest that androgens could act to modulate (reduce) bone turnover in response to these agents.

Finally, both androgen (Hofbauer and Khosla, 1999) and estrogen (Kassem *et al.,* 1996; Passeri *et al.,* 1993) (but see Rifas *et al.,* 1995) inhibit production of interleukin-6 by osteoblastic cells. In stromal cells of the bone marrow, androgens have been shown to have potent inhibitory effects on the production of interleukin-6 and the subsequent stimulation of osteoclastogenesis by marrow osteoclast precursors (Bellido *et al.,* 1995). Adrenal androgens (androstenediol, androstenedione, DHEA) have similar inhibitory activities on interleukin-6 gene expression and protein production by stromal cells (Bellido *et al.,* 1995). The loss of inhibition of interleukin-6 production by androgen may also contribute to the marked increase in bone remodeling and resorption that follows orchiectomy, in addition to modulation of osteoclast activity through changes in the OPG/RANKL ratio as noted earlier. Moreover, androgens inhibit the expression of the genes encoding the two subunits of the IL-6 receptor (gp80 and gp130) in the murine bone marrow, another mechanism that may blunt the effects of this osteoclastogenic cytokine in intact animals (Lin *et al.,* 1997). In these aspects, the effects of androgens seem to be similar to those of estrogen, which may also indirectly inhibit osteoclastogenesis via mechanisms that involve interleukin-6 inhibition and/or OPG/RANKL ratio changes.

The Skeletal Effects of Androgen: Animal Studies

The effects of androgens on bone remodeling have been examined fairly extensively in animal models. Much of this work has been in species such as rodents, not perfectly suited to reflect human bone metabolism (but see Kalu, 1991), and certainly the field remains incompletely

explored. Nevertheless, animal models do provide valuable insights into the effects of androgens at organ and cellular levels. Many of the studies of androgen action have been performed in male rats, in which rapid skeletal growth occurs until about four months of age, at which time epiphyseal growth slows markedly (although never completely ceases at some sites). Because the effects of androgen deficiency may be different in growing versus more mature adult animals (see Vanderschueren *et al.,* 2004), it is appropriate to consider the two situations independently.

Effects on Epiphyseal Function and Bone Growth during Skeletal Development and Puberty

In most mammals there is a marked gender difference in bone morphology. The mechanisms responsible for these differences are complex, and presumably involve both androgenic and estrogenic actions. Estrogens are particularly important for the regulation of epiphyseal function, and act to reduce the rate of longitudinal growth via influences on chondrocyte proliferation and action, as well as on the timing of epiphyseal closure (Turner *et al.,* 1994). Androgens appear to have opposite effects, and tend to promote long bone growth, chondrocyte maturation, and metaphyseal ossification. Androgen deficiency retards those processes (Lebovitz and Eisenbarth, 1975). Excess concentrations of androgen will accelerate aging of the growth plate and reduce growth potential (Iannotti, 1990), possibly via conversion to estrogens.

Although the specific roles of sex steroids in the regulation of epiphyseal growth and maturation remain somewhat unresolved, there is evidence that androgens do have direct effects independent of those of estrogen. For instance, testosterone injected directly into the growth plates of rats increases plate width (Ren *et al.,* 1989). In a model of endochondral bone development based on the subcutaneous implantation of demineralized bone matrix in castrate rats, both testosterone and DHT increase the incorporation of calcium during osteoid formation (Kapur and Reddi, 1989). Interestingly, in this model androgens reduced the incorporation of [^{35}S]sulfate into glycosaminoglycans early in the developing cartilage. In sum, these data support the contention that androgens play a direct role in chondrocyte physiology, but how these actions are integrated with those of other regulators is unclear.

During childhood and adolescence, skeletal development is characterized by marked expansion of cortical proportions and increasing trabecular density. During this process, the skeleton develops distinctly in males and females, most significantly at the periosteal surface. Thus, sex differences in skeletal morphology and physiology occur at or around puberty. For that reason, it is hypothesized that gender differences, particularly with respect to "bone quality" and

architecture, i.e., predominantly bone width, are modulated by the sex steroids estrogen and androgen. Consistent with this, a distinct response to estrogen and androgen has been described *in vivo* especially in cortical bone. At the periosteum, estrogen suppresses while androgen stimulates new bone formation (Matsumoto *et al.,* 2006; Wiren *et al.,* 2004b), yet conversely at the endosteal surface estrogen stimulates but androgen strongly suppresses formation (Wiren *et al.,* 2004b). Thus, estrogen decreases but androgen increases radial growth in cortical bone through periosteal apposition, indicating that these two sex steroids may act in opposition in some situations at distinct bone compartments. These divergent responses to estrogen and androgen during growth likely play an important role in determining sexual dimorphism of the skeleton, i.e., that male bones are wider but not thicker than females (Seeman, 2003). Young men do have larger bone areas than women with increased whole bone cross-sectional area, particularly at peripheral sites (Riggs *et al.,* 2004). Low levels of estrogen in the obligate presence of androgen and AR may also be important for stimulation of periosteal bone formation during development (Bouillon *et al.,* 2004; Rochira *et al.,* 2007), but estrogen is not apparently important after puberty (Matsumoto *et al.,* 2006). Sex steroid effects can be influenced by interaction with the growth hormone IGF-I axis in the coordination of skeletal growth. Growth hormone deficiency in males has no net effect on endosteal growth but reduced by half expansion at the periosteal surface (Kim *et al.,* 2003), and produces greater deficits in females. The effect of growth hormone on periosteal formation may be mediated by increased IGF-I concentrations (Venken *et al.,* 2007). Although androgens did not affect IGF-I expression, both androgen and IGF-I were required for optimal stimulation at the periosteal surface. In contrast, analysis indicates that androgens stimulate trabecular bone modeling independent of growth hormone. Androgens are also essential for the production of peak total-body bone mass in males (Vanderschueren *et al.,* 2005), and a recent study has found that serum testosterone and IGF-1/IGFBP-3 ratio are major determinants of BMD at different stages of puberty in males (Pomerants *et al.,* 2007).

Mature Male Animals

Results from animal studies also support an effect of androgen on bone formation in the mature animal. Experimental strategies such as pharmacological or surgical (gonadectomy) intervention and examination of genetic models have all been employed to characterize androgen signaling in the adult. As expected, the consequences of either androgen administration or conversely withdrawal are complex, with effects in all bone compartments (see Fig. 3). In mature rats, castration eventually results in osteopenia and both cortical and trabecular compartments are affected. At a time when longitudinal growth has slowed markedly,
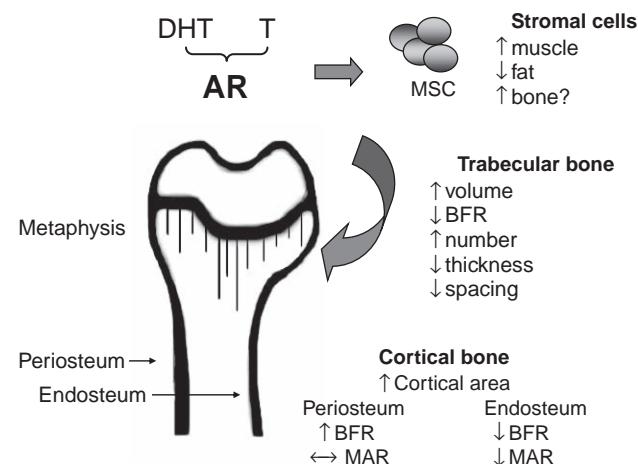
FIGURE 3 Model for androgen-mediated action in the skeleton, derived from androgen deficient or androgen replacement studies. References are cited in the text. AR activation by androgen influences a variety of skeletal compartments or target sites, including stromal cells, trabecular bone, intramembranous bone (not shown), and cortical bone. Arrows indicate the changes induced by androgen action. In general, AR activation in bone cells results in a low turnover phenotype and may also influence body composition.

pronounced differences as a consequence of castration appear in cortical bone ash weight per unit length, cross-sectional area, cortical thickness, and bone mineral density (Danielsen, 1992; Vanderschueren *et al.,* 1992; Verhas *et al.,* 1986; Wink and Felts, 1980). Distinct effects of androgen are seen with gonadectomy when comparing the effects of orchiectomy in male vs. ovariectomy in female rats. Generally castration results in changes in both trabecular and cortical bone compartments, and dramatic bone loss in trabecular bone is noted in both males and females, but sex-specific responses are most dimorphic in cortical bone. Ovariectomy and the associated loss of sex steroids in the female generally results in decreased trabecular area with increased osteoclast number. In cortical bone in ovariectomized females, an increase at the periosteal surface is seen with circumferential enlargement but a decrease in endosteal labeling. These results demonstrate that estrogen protects trabecular bone predominantly through inhibition of osteoclast activity/recruitment, but has an inhibitory action at the periosteal surface as noted earlier (for example, see Vandenput *et al.,* 2004). In the male, orchiectomy with the attendant loss of sex steroids also results in decreased trabecular area with increased osteoclast number as in females. However, careful histomorphometric analysis of androgen action in orchiectomized male mice has shown that the bone-sparing effect of AR activation in trabecular bone is distinct from a similar bone-sparing effect of ERα at that site (Moverare *et al.,* 2003). The analysis showed that AR activation does preserve the number of trabeculae, but does not preserve thickness, volumetric density, nor mechanical strength in cortical bone. In cortical bone,

periosteal formation is reduced with the loss of androgen after gonadectomy in males, in contrast to ovariectomized females. Androgen treatment is effective in suppressing the acceleration of bone remodeling normally seen after orchiectomy (Venken *et al.,* 2005a). This divergent response at the periosteal surface after castration in male and female animals abolishes the sexual dimorphism usually present in radial bone growth. In the intact animal, the stimulation of endosteal formation by estrogen compensates for the lack of periosteal formation, thus leading to no difference in cortical width between the sexes. Nevertheless, factors that influence periosteal apposition may constitute an important therapeutic class because periosteal bone formation is often a neglected determinant of bone strength (Seeman, 2003). Orchiectomy shows little net effect on the endosteal surface in males (Kim *et al.,* 2003) or slight reductions likely due to increased resorption. Consistent with this, increased intracortical resorption cavities are reported to result from orchiectomy (Prakasam *et al.,* 1999; Wink and Felts, 1980). As might be expected in light of these changes, breaking strength (N) is decreased in cortical bone (Kim *et al.,* 2003). Interestingly, DHT as a specific AR agonist is less effective than testosterone in the cortical bone compartment in elderly orchidectomized rats (Vandenput *et al.,* 2002; Wakley *et al.,* 1991). In addition, it appears that orchiectomy affects cranial development more than ovariectomy (Fujita *et al.,* 2004), suggesting that androgen action is particularly important in intramembranous bone.

Changes in the skeleton can occur rapidly after castration, and osteopenia becomes pronounced with time. This bone loss appears to result in part from increased bone resorption, as it is associated with increases in resorption cavities, osteoclasts, and blood flow (Verhas *et al.,* 1986; Wink and Felts, 1980). Initially, dynamic histomorphometric and biochemical measures of bone remodeling increase quickly, with evidence of increased osteoclast numbers only one week after castration (Gunness and Orwoll, 1995; Vanderschueren *et al.,* 1994a; Wink and Felts, 1980). Changes include an increase in osteoblastic activity as well as increased bone resorption, reflecting an initial high turnover state that is followed by a reduction in remodeling rates and low turnover osteopenia. In the SAMP6 mouse, a model of accelerated senescence in which osteoblastic function is impaired, the rise in remodeling following orchiectomy is blunted, which has been interpreted as evidence that the early changes after gonadectomy are dependent on osteoblast-derived signals (Weinstein *et al.,* 1997). As noted earlier, androgens reduce osteoclast formation and activity (Huber *et al.,* 2001), which may be partially indirectly mediated by increased OPG levels (Chen *et al.,* 2004; Wiren *et al.,* 2004b). The initial phase of increased bone remodeling activity subsides with time (Vanderschueren *et al.,* 1994a; Verhas *et al.,* 1986) and by four months there is evidence of a depression in bone turnover rates in some skeletal areas. As in younger animals,

indices of mineral metabolism are not altered by these changes in skeletal metabolism (Vanderschueren *et al.,* 1992).

As a potential model for the effects of hypogonadism in humans (see Vanderschueren *et al.,* 2004), animal models therefore indicate an early phase of high bone turnover and bone loss after orchiectomy, followed by a reduction in remodeling rates and osteopenia. The remodeling imbalance responsible for loss of bone mass appears complex, as there are changes in rates of both bone formation and resorption, and patterns that vary from one skeletal compartment to another. Broad changes are similar (but not identical in detail) to those noted in female animals after castration, in which a loss of estrogen signaling has been associated with a stimulation of osteoblast progenitor differentiation, an increase in osteoclast numbers, bone resorption, and bone loss (Jilka *et al.,* 1998).

Androgens in the Female Animal

Of course androgens are present in both sexes and likely also affect bone metabolism in females. Although testosterone serum concentrations are much lower in females than in males, the concentration of other androgens like androstenedione and DHEA-sulfate are in fact similar between the sexes. In castrated female rats, DHT administration suppresses elevated concentrations of osteocalcin and of bone resorption markers (Mason and Morris, 1997). However, alkaline phosphatase activity increases further. Additional evidence to support the contention that androgens play a role in females includes the fact that antiandrogens are capable of evoking osteopenia in intact (i.e., fully estrogenized) female rats (Goulding and Gold, 1993; Lea *et al.,* 1996). This finding suggests that androgens provide crucial support to bone mass in females, in addition to a role for estrogens. Of interest, the character of the bone loss induced by flutamide suggested that estrogen prevents bone resorption whereas androgens may stimulate bone formation. In periosteal bone, DHT and testosterone stimulate periosteal formation after orchiectomy in young male rats, whereas in castrated females they suppress bone formation (Turner *et al.,* 1990b), perhaps reflecting an interaction or synergism between sex steroids and their effects on bone. However, in ovariectomized females with established osteopenia, treatment with DHT-stimulated periosteal bone formation whereas estradiol inhibited formation at the same surface (Coxam *et al.,* 1996). As noted earlier, combination therapy with estrogen and androgen in postmenopausal women is more beneficial than either steroid alone (Castelo-Branco *et al.,* 2000; Miller *et al.,* 2000; Raisz *et al.,* 1996), which has been confirmed in an animal model (Tivesten *et al.,* 2004). There is also some information concerning androgens in additional animal models, including primates. For instance,

in young female cynomolgus monkeys, testosterone treatment increased cortical and trabecular bone density as well as biomechanical strength (Kasra and Grynpas, 1995).

Gender Effects

In most mammals, there is a marked gender difference in morphology that results in a sexually dimorphic skeleton. The mechanisms responsible for these differences are obviously complex, and presumably involve both androgenic and estrogenic actions on the skeleton linked to different levels of each steroid between males and females. It is becoming increasingly clear that estrogens are particularly important for the regulation of epiphyseal function and act to reduce the rate of longitudinal growth via influences on chondrocyte proliferation and function, as well as on the timing of epiphyseal closure (Turner *et al.,* 1994). Androgens, on the other hand, appear to have opposite effects to estrogen on the skeleton. Androgens tend to promote long bone growth, chondrocyte maturation, and metaphyseal ossification as noted earlier (Cassorla *et al.,* 1984). Furthermore, the most dramatic effect of androgens is likely on bone size, consistent with gender-specific effects of androgens on periosteal bone formation (Turner *et al.,* 1990b). This difference of course has important biomechanical implications, with thicker bones being stronger bones (Seeman, 2003). Consistent with gender specificity, the response of the adult skeleton to the same intervention results is distinct responses in males and females. For example, in a model of disuse osteopenia, antiorthostatic suspension results in significant reduction in bone formation rate at the endosteal perimeter in males. In females, however, a decrease in bone formation rate occurred along the periosteal perimeter (Bateman *et al.,* 1997). However, DHT has also been shown to enhance periosteal bone formation after ovariectomy in females with established osteopenia (Coxam *et al.,* 1996), similar to the response in males noted earlier. Nevertheless, gender-specific responses *in vivo* and *in vitro* (for example, see Somjen *et al.,* 2006), and the mechanism(s) that underlie such responses in bone cells, may have implications in treatment options for metabolic bone disease.

ANIMAL MODELS OF ALTERED ANDROGEN RESPONSIVENESS

The specific contribution of AR signaling *in vivo* has also been approached in genetic animal models with global AR modulation, including the testicular feminization (Tfm) model of androgen insensitivity (Tozum *et al.,* 2004; Vandenput *et al.,* 2004) and with nontargeted (global) AR knockout mice (Kawano *et al.,* 2003; Yeh *et al.,* 2002). The Tfm male rat is AR signaling deficient, and provides an interesting model for the study of the unique effects of

androgens in bone. In these rats androgens are generally incapable of action at the AR, but the model is complicated by the fact that estrogen and androstenedione concentrations are considerably higher than in normal males (Vanderschueren *et al.,* 1994b; Vanderschueren *et al.,* 1993b). Clear increases also exist in Tfm male rats in serum concentrations of osteocalcin, calcium, and phosphorus, whereas IGF-1 concentrations are decreased. Estimates of bone mass suggest that Tfm rats have reduced longitudinal and radial growth rates, but trabecular volume and density are similar to those of normal rats, likely a consequence of high serum estrogen levels. In selected sites, measures of bone mass and remodeling were intermediate between normal male and female values. With castration, bone volume is markedly reduced in Tfm male rats, suggesting a major role for estrogens as well in skeletal homeostasis. This model indicates that androgens do have an independent role to play in normal bone growth and metabolism, but the model as described is complex and not easily dissected. Tfm mice are different from Tfm rats in that Tfm mice develop a high-turnover trabecular bone phenotype (Vandenput *et al.,* 2004), associated with low levels of circulating testosterone and estradiol. Meticulous analysis in Tfm mice has shown that the positive effects of testosterone on cortical bone are generally mediated by stimulation of periosteal bone formation through the AR (Vandenput *et al.,* 2004), whereas testosterone effects on trabecular bone likely involves both AR and ER signaling. Thus, these studies demonstrate that AR-mediated testosterone action is essential for periosteal bone formation (in male mice), while AR contributes to trabecular bone maintenance along with ER. This is very similar to the study of humans with the androgen insensitivity syndrome. Even when compliance with estrogen replacement is excellent, there is a deficit in bone mineral density in women with androgen insensitivity (Marcus *et al.,* 2000) that has been observed at the spine (Danilovic *et al.,* 2007) or at both spine and hip sites (Sobel *et al.,* 2006). In addition, final height was intermediate between what would be predicted for males or males (Danilovic *et al.,* 2007). These results provide evidence for an important role for androgens in normal male growth and bone density that is not replaced by estrogens. However, inadequate estrogen replacement appeared to worsen the deficit (Marcus *et al.,* 2000), and other environmental factors are difficult to quantitate. Thus, in Tfm models that lack functional AR, orchiectomy demonstrates the importance of AR in mediating the positive effects of androgen to contribute to trabecular bone maintenance, and in cortical bone particularly at the periosteal surface (Tozum *et al.,* 2004; Vandenput *et al.,* 2004).

Global AR null mice, developed using a cre/loxP approach, have complete AR disruption from birth (De Gendt *et al.,* 2005; Sato *et al.,* 2002; Yeh *et al.,* 2002). The ARKO null model should not be confused with the aromatase null, or ArKO, mouse. The bone phenotype that

develops in male ARKO mice is a high-turnover osteopenia, with reduced trabecular bone volume and a significant stimulatory effect on osteoclast function (Kato *et al.,* 2004; Kawano *et al.,* 2003; Venken *et al.,* 2006; Yeh *et al.,* 2002). As expected, bone loss with orchiectomy in male ARKO mice was only partially prevented by treatment with aromatizable testosterone due to the lack of AR function. Interestingly, orchiectomy in control mice produced a similar trabecular bone phenotype to that observed in the ARKO male, indicating that AR activation is a major determinant of trabecular bone development (Venken *et al.,* 2006). Analysis of the impact of androgens with and without aromatase inhibitors suggests that aromatase inhibition of periosteal bone formation is dependent on AR expression, as aromatase treatment was without effect in ARKO mice but reduced the effect of androgen to stimulate periosteal apposition in controls (Venken *et al.,* 2006). In contrast, aromatase inhibition had no effect on protection of trabecular bone by either testosterone or DHT after orchiectomy, similar to results obtained in Tfm mice described earlier.

Final models for AR modulation are represented by generation of mice with skeletally targeted modification of AR expression, with either overexpression (Wiren *et al.,* 2004b) or deletion using a tissue-specific cre/loxP approach (Notini *et al.,* 2007). In the first model, full-length AR is under the control of the 3.6 kb type I collagen promoter, with AR overexpression in osteoblast stromal precursors and throughout the osteoblast lineage. AR3.6-transgenic mice are the only model with bone-targeted overexpression of AR, and demonstrate enhanced sensitivity to androgen in tissues where AR is overexpressed yet without changes in circulating steroids or the complication of systemic androgen administration (Wiren *et al.,* 2004b). AR overexpression in this model results in a complex phenotype predominantly in males, with increased trabecular bone mass (with increased trabecular number but not thickness) in the setting of inhibition of resorption due to reduced osteoclast activity. In addition, cortical formation is altered in an envelope-specific fashion, with periosteal expansion but inhibition of inner endosteal deposition, in line with the known effects of androgen to stimulate periosteal apposition and opposite to the effects of estrogen on these compartments. Inhibition of osteoclastic resorption may be responsible for altered trabecular morphology, consistent with reduced osteoclast activity and increased trabecular bone volume observed with androgen therapy in rodents and humans. In trabecular bone, AR signaling results in increased volume, increased trabecular number, and decreased spacing with a modest reduction of thickness. The dramatic inhibition of bone formation at the endosteal envelope may underlie the modest decrease in cortical bone area and subsequent reductions in biomechanical properties observed. A second model for specific bone targeting results in disruption of normal AR expression using col

2.3-cre mice crossed with floxed AR mice (Notini *et al.,* 2007). Knockdown of AR expression is confined to mature osteoblasts/osteocytes. During development, there is a progressive loss of trabecular bone with decreased trabecular number but increased spacing and increased width, opposite to the phenotype observed with AR overexpression. There was little effect on cortical bone in this model. Notably, the bone phenotype observed in these models is consistent with many of the known effects of androgen treatment on the skeleton. Combined, these results indicate that AR expressed in bone can be a direct mediator of androgen action to influence skeletal development and homeostasis.

EFFECTS ON THE PERIOSTEUM: THE ROLE OF AR VS. AROMATIZATION OF TESTOSTERONE

As noted earlier, androgen-mediated AR transactivation is likely a key determinant of the sexually dimorphic pattern of periosteal apposition, an effect that is clearly demonstrated in male AR3.6-transgenic mice even in the absence of hormone administration (Wiren *et al.,* 2004b). Furthermore, essentially all of the alterations induced by orchiectomy (in both growing and mature animals) can be prevented at least in part by replacement with either testosterone or nonaromatizable androgens (Kapitola *et al.,* 1995; Prakasam *et al.,* 1999; Schoutens *et al.,* 1984; Somjen *et al.,* 1994; Turner *et al.,* 1990b; Vanderschueren *et al.,* 1993a; Wakley *et al.,* 1991). In sum, these results strongly suggest that aromatization of androgens to estrogens cannot fully explain the actions of androgens on bone metabolism.

However estrogens also seem to play a role in the effects of androgen on apposition, likely through indirect mechanisms. Although AR activity is essential, low levels of estrogens are likely required for optimal stimulation of periosteal growth (Venken *et al.,* 2006), as observed in aromatase deficiency even in males (Bouillon *et al.,* 2004). Estrogens may also help prevent bone loss following castration in male animals. For example, it has been reported (Vanderschueren *et al.,* 1992) that estradiol (and also nandrolone) was capable of not only preventing the increase in biochemical indices stimulated by orchiectomy, but also preventing cortical and trabecular bone loss. In fact, estradiol resulted in an absolute increase in trabecular bone volume not achieved with androgen replacement. Many of these estrogen-mediated responses appear to be indirectly mediated through increased IGF-I levels (Venken *et al.,* 2005b); in contrast androgens stimulate periosteal apposition independent of the growth hormone -IGF axis as noted earlier (Venken *et al.,* 2007). Similarly, estrogen was reported to antagonize the increase in blood flow resulting from castration and to increase bone ash weight more consistently than testosterone.

The gender reversal of androgen administration to female animals is also instructive. Consistent with the stimulatory effect of androgen on the periosteal compartment, treatment with nonaromatizable DHT has been shown to increase periosteal bone formation in ovariectomized females with established osteopenia (Coxam *et al.,* 1996). Nonaromatizable androgens are also capable of preventing or reversing osteopenia and abnormalities in bone remodeling in ovariectomized females (Tobias *et al.,* 1994; Turner *et al.,* 1990b). These actions result from the suppression of trabecular bone resorption as well as stimulation of periosteal bone formation (Tobias *et al.,* 1994). Very similar results have been reported following the treatment of ovariectomized animals with DHEA (Turner *et al.,* 1990b). Moreover, blockage of androgen action with an AR antagonist in female rats already treated with an estrogen antagonist increases bone loss and indices of osteoclast activity more than treatment with an estrogen antagonist alone (Lea and Flanagan, 1999), again indicating that ovarian androgens (apart from estrogens) exert a protective effect on bone in females. Analogously, androstenedione reduces (although does not abrogate) trabecular bone loss and remodeling alterations in ovariectomized animals treated with an aromatase inhibitor. This protective effect was blocked by the addition of an AR antagonist (Lea and Flanagan, 1998; Lea *et al.,* 1998). Finally, although aromatase inhibition in male rats reduces bone mass, the large increase in remodeling induced by orchiectomy does not occur in these animals (Vanderschueren *et al.,* 1997). Also, orchiectomy in ERα null (α-ERKO) mice further reduces bone mass (Sims *et al.,* 2003). The latter observation implicates a role for androgens in the maintenance of bone mass in α-ERKO mice.

SUMMARY

The effects of androgens on bone health are obviously pervasive and complex (see Fig. 3). Androgens are important in the maintenance of a healthy skeleton, and have been shown to stimulate bone formation in the periosteum but reduce formation on the endosteal surface in cortical bone and in trabecular bone and reduce osteoclast activity. Androgens influence skeletal modeling and remolding by multiple mechanisms through effects on osteoblasts, osteoclasts, and even perhaps an influence on the differentiation of pluripotent stem cells toward distinct lineages. The specific effects of androgen on bone cells are mediated directly through an AR-signaling pathway, but there are also indirect contributions to overall skeletal health through aromatization and ER signaling. The effects of androgens are particularly dramatic during growth in boys, but almost certainly play an important role during this period in girls as well. Throughout the rest of life, androgens affect skeletal function in both sexes. Still poorly characterized, more

needs to be done to unravel the mechanisms by which androgens influence the physiology and pathophysiology of bone, and there remains much to be learned about the roles of androgens at all levels. The interaction of androgens and estrogens, and how their respective actions in the skeleton and in other tissues can be utilized for specific diagnostic and therapeutic benefit, are important but unanswered issues. With an increase in the understanding of the nature of androgen effects will come greater opportunities to use their positive actions in the prevention and treatment of a wide variety of skeletal disorders.

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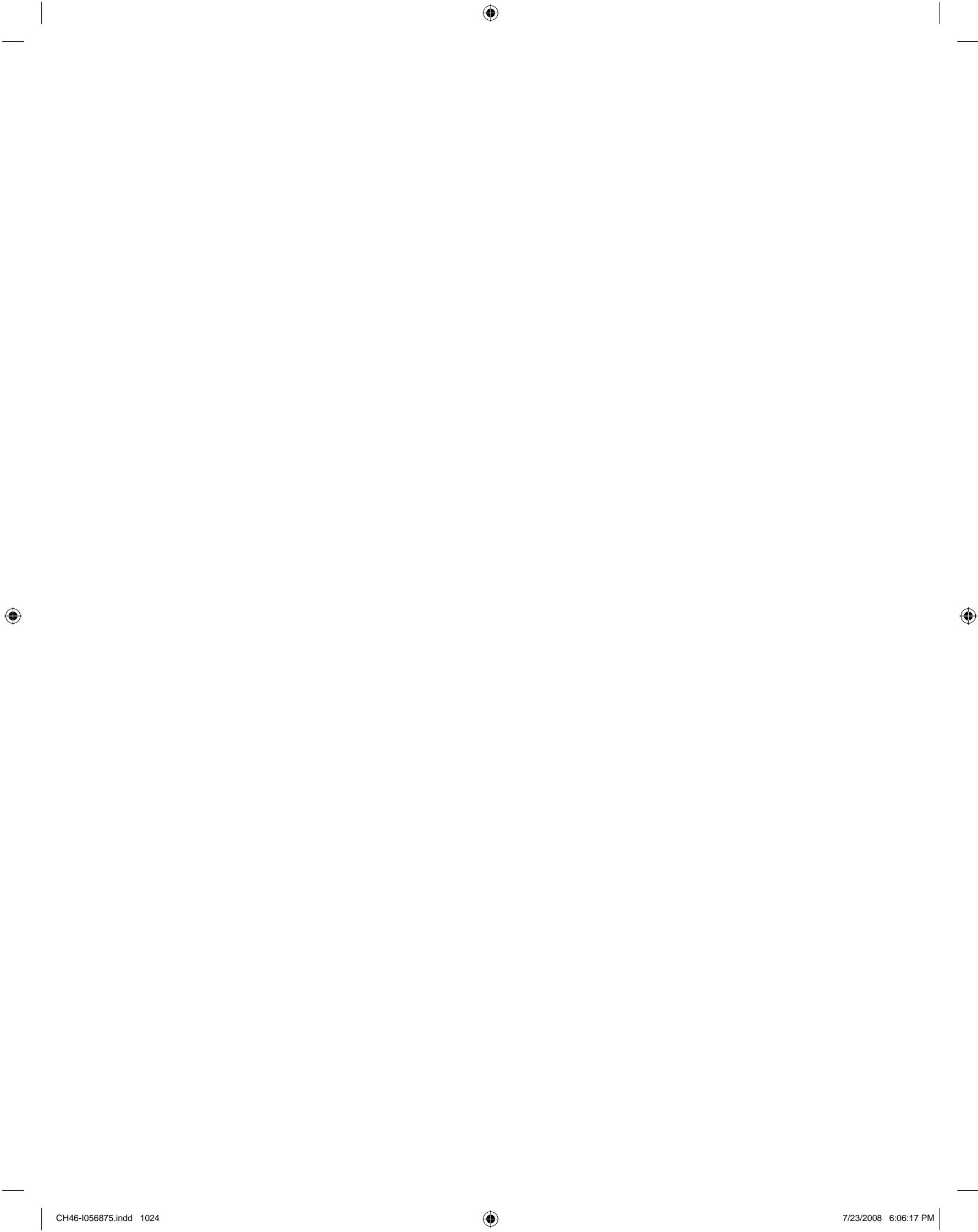
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Abstract	The obvious impact of the menopause on skeletal health has focused much of the research describing the general action of gonadal steroids on the specific effects of estrogen in bone. However, androgens clearly have important beneficial effects, in both men and women, on skeletal development and on the maintenance of bone mass *(1,2)*.

Keywords (separated by '-')	androgen - androgen receptor - osteoblast - osteoclast - bone

16 Androgen Action in Bone: Basic Cellular and Molecular Aspects

Kristine M. Wiren, PhD

CONTENTS

AQ1

Key Words: androgen, androgen receptor, osteoblast, osteoclast, bone

INTRODUCTION

The obvious impact of the menopause on skeletal health has focused much of the research describing the general action of gonadal steroids on the specific effects of estrogen in bone. However, androgens clearly have important beneficial effects, in both men and women, on skeletal development and on the maintenance of bone mass *(1,2)*. Thus it has been demonstrated that androgens (a) influence growth plate maturation and closure helping to determine longitudinal bone growth during development, (b) mediate regulation of trabecular (cancellous) and cortical bone mass in a fashion distinct from estrogen, leading to a sexually dimorphic skeleton, (c) modulate peak bone mass acquisition, and (d) inhibit bone loss *(2)*. In castrate animals, replacement with nonaromatizable androgens (e.g., 5α-dihydrotestosterone, DHT) yields beneficial effects that are clearly distinct from those observed with estrogen replacement *(3,4)*. In intact females, blockade of the androgen receptor (AR) with the specific AR antagonist hydroxyflutamide results in osteopenia *(5)*. Furthermore, treatment with nonaromatizable androgen alone in females results in improvements in bone mineral density *(6)*. Finally, combination therapy with estrogen and androgen in postmenopausal women is more beneficial than either steroid alone *(7–9)*, indicating non-parallel and distinct pathways of action. Combined, these reports illustrate the distinct

47 actions of androgens and estrogens on the skeleton. Thus, in both men and women it is
48 probable that androgens and estrogens each have important yet distinct functions during
49 bone development, and in the subsequent maintenance of skeletal homeostasis in the adult.
50 With the awakening awareness of the importance of the effects of androgen on skeletal
51 homeostasis, and the potential to make use of this information for the treatment of bone
52 disorders, much remains to be learned.

MOLECULAR MECHANISMS OF ANDROGEN ACTION IN BONE CELLS: THE ANDROGEN RECEPTOR (AR)

57 Direct characterization of AR expression in a variety of tissues, including bone *(10)*,
58 was made possible by the cloning of the AR cDNA *(11,12)*. The AR is a member of
59 the class I (the so-called classical or steroid) nuclear receptor superfamily, as are the
60 (estrogen receptor) ERα and ERβ isoforms, the progesterone receptor, the mineralocor-
61 ticoid, and glucocorticoid receptor *(13)*. Steroid receptors are transcription factors with
62 a highly conserved modular design characterized by three functional domains: the trans-
63 activation, DNA binding, and ligand binding domains. In the absence of ligand, the AR
64 protein is generally localized in the cytoplasmic compartment of target cells in a large
65 complex of molecular chaperones, consisting of loosely bound heat-shock, cyclophilin,
66 and other accessory proteins *(14)*. Interestingly, in the unliganded form, AR conforma-
67 tion is unique with a relatively unstructured amino-terminal transactivation domain *(15)*.
68 As lipids, androgens can freely diffuse through the plasma membrane to bind the AR to
69 induce a conformational change. Once bound by ligand, the AR dissociates from the mul-
70 tiprotein complex, translocates to the nucleus, and recruits coactivators or corepressors that
71 can display cell-type specific expression *(16)*, allowing the formation of homodimers (or
72 potentially heterodimers) that activate a cascade of events in the nucleus *(17)*. Bound to
73 DNA, the AR influences transcription and/or translation of a specific network of genes,
74 leading to the cellular response to the steroid.

The Androgen Receptor Signaling Pathway

77 Once bound by ligand, the AR is activated. As shown in Fig. 1, this allows the forma-
78 tion of homodimers (or potentially heterodimers) that bind to DNA at palindromic androgen
79 response elements (AREs) in androgen-responsive gene promoters. Classic ARE sequences
80 are found in the proximal promoter as a motif represented by an inverted repeat separated by
81 3 bp *(18)* similar to glucocorticoid response elements *(19)*. However, our understanding of
82 hormone binding sites in DNA is becoming better characterized and is more complex than
83 originally described *(20)*. Thus, AR binding sites that influence expression, both positively
84 and negatively, are likely distributed throughout the genome with sequences more complex
85 and diverse than simple ARE repeats. DNA binding of the activated AR organizes a cas-
86 cade of events in the nucleus leading to transcription and translation of a specific network
87 of genes that is responsible for the cellular response to the steroid *(17)*. In the classic model
88 of steroid action, the latent receptor is converted into a transcriptionally active form by
89 simple ligand binding. Again, this model is now considered an over-simplification, with the
90 understanding that signaling pathways and additional proteins (for example, coactivators or
91 corepressors as described below and shown in Fig. 1) within the cell can influence steroid
92 receptor transduction activity. Furthermore, posttranslational modification of the receptor
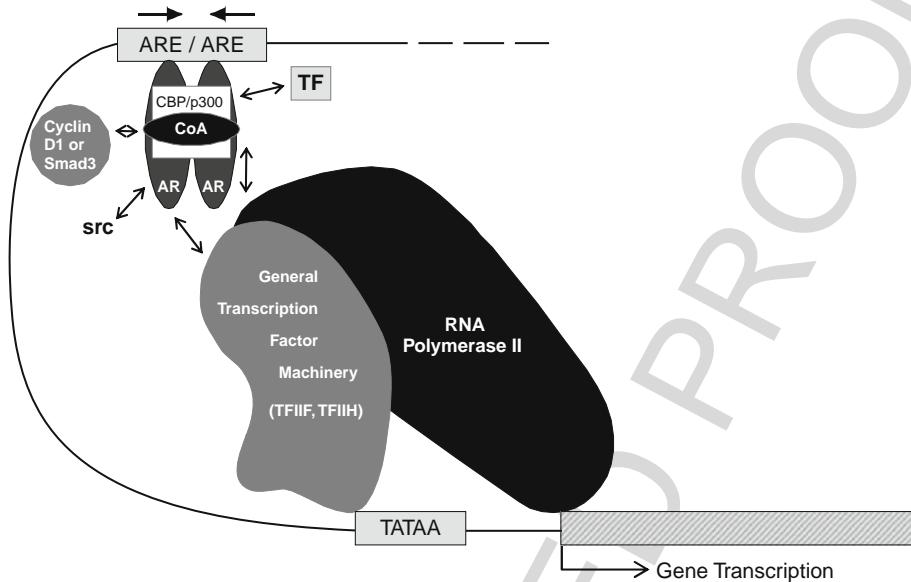
Fig. 1. Model of AR regulation of gene expression. Binding of androgen promotes high-affinity dimerization, followed by DNA binding at the androgen response element (ARE) in an androgen-responsive gene promoter. Coactivators may remodel chromatin through histone acetylase activity to open chromatin structure *(157)* or act as a bridge to attract transcription factors (TFs) that target binding of TATA-binding protein to the TATAA sequence *(13)*. Phosphorylation of receptor may result from activation of SRC by growth factors *(22)*. Smad3 can act as either a coactivator or a corepressor *(158,159)*, while cyclin D1 is a corepressor of AR transactivation *(21)*. AR can also directly contact TFIIH and TFIIF *(160)* in the general transcription machinery. Such interactions between the AR and the general transcription machinery, leading to stable assembly, result in recruitment of RNA polymerase II and subsequent increased gene transcription. Downregulation of gene expression can also be AR mediated.

by acetylation, phosphorylation, and/or ubiquitination can occur *(21)*. For example, steroid receptor phosphorylation can result from signal transduction cascades initiated at the cell membrane, such as from activation of src kinases by growth factors *(22)*. It has been shown that steroid receptor phosphorylation can lead to alterations of the responsiveness of steroid receptors to cognate ligands or, in some cases, even result in ligand-independent activation.

Such potential modification(s) of AR action in bone cells is only poorly characterized; whether the AR in osteoblasts undergoes posttranslational processing that might influence AR activity as described in other tissues *(23,24)*, and the potential functional implications *(25,26)*, are also unknown. Ligand-independent activation of AR has also been described in other tissues *(27)*, but has not been explored in bone. AR activity may also be influenced by receptor modulators, such as the nuclear receptor coactivators or corepressors *(22,28,29)*. These coactivators/corepressors can influence the downstream signaling of nuclear receptors through multiple mechanisms, including histone acetylation/deacetylation, respectively, that result in chromatin remodeling. Such activities may reflect both the cellular context and the particular promoter involved. AR-specific coactivators have been identified *(30)*, many of which interact with the ligand-binding domain of the receptor *(31)*. Expression and regulation of these modulators may thus influence

139 the ability of steroid receptors to regulate gene expression in bone *(18)*, but this remains
140 underexplored with respect to androgen action. The specific coactivator/corepressor profile
141 present in cells representing different bone compartments (i.e., periosteal cells, proliferat-
142 ing or mineralizing cells) may help to determine the activity of selective AR modulators
143 (SARMS) as described below that influence transcriptional activity of the AR.
144   The number of specific androgen binding sites in osteoblasts varies, depending on the
145 methodology and the cell source, from 1000 to 14,000 sites/cell *(32–35)*, but is in a range
146 seen in other androgen target tissues. Furthermore, the binding affinity of the AR found in
147 osteoblastic cells ($K_d = 0.5$–2×10^{-9}) is typical of that found in other tissues. Androgen
148 binding is specific, without significant competition by estrogen, progesterone, or dexam-
149 ethasone *(33,35,36)*. Finally, testosterone and DHT appear to have relatively similar binding
150 affinities *(33,37)*. All these data are consistent with the notion that the direct biologic effects
151 of androgenic steroids in osteoblasts are mediated at least in part via classic mechanisms
152 associated with the AR.
153   In addition to the classical AR present in bone cells, several other androgen-dependent
154 signaling pathways have been described. Specific binding sites for weaker adrenal andro-
155 gens (such as dehydroepiandrosterone, DHEA) have been described *(38)*; DHEA can also
156 transactivate AR *(39)*, thus raising the possibility that DHEA or similar androgenic com-
157 pounds may also have direct effects in bone. DHEA and its metabolites may also bind and
158 activate additional receptors, including ER, peroxisome proliferator-activated receptor-α,
159 and pregnane X receptor *(40)*. Bodine et al. *(41)* showed that DHEA caused a rapid inhibi-
160 tion of c-*fos* expression in human osteoblastic cells that was more robust than seen with the
161 classical androgens (DHT, testosterone, androstenedione). In addition, DHEA may inhibit
162 bone resorption by osteoclasts when in the presence of osteoblasts, likely through changes
163 in osteoprotegerin (OPG) and receptor activator of NF-κB ligand (RANKL) concentrations
164 *(42)*. AR may also interact with other transcription factors, such as NF-κB, CREB-binding
165 protein, and different forms of AP-1, to generally repress transcription without DNA bind-
166 ing. Alternatively, androgens may be specifically bound in osteoblastic cells by a novel
167 63-kDa cytosolic protein *(43)*. In addition, there are reports of distinct AR polymorphisms
168 identified in different races that may have biological impact on androgen responses *(44)*, but
169 to date none have an effect with respect to bone tissue *(45)*. These different isoforms have
170 the potential to interact in distinct fashions with other signaling molecules, such as c-Jun
171 *(46)*. Finally, androgens may regulate osteoblast activity via rapid nongenomic mecha-
172 nisms *(47,48)* through membrane receptors displayed at the bone cell surface *(49)*. The role
173 and biologic significance of these non-classical signaling pathways in androgen-mediated
174 responses in bone remains controversial, and most data suggest that genomic signaling may
175 be the more significant regulator in bone and other tissues *(50–53)*.

176
177   *Localization of Androgen Receptor Expression*

178   Clues about the potential sequelae of AR signaling may be derived from a better
179 understanding of the cell types in which receptor expression is documented. In the bone
180 microenvironment, the localization of AR expression in osteoblasts has been described
181 in intact human bone by using immunocytochemical techniques *(10,54)*. In developing
182 bone from young adults, Abu et al. *(10)* showed ARs were predominantly expressed in
183 active osteoblasts at sites of bone formation (Fig. 2). ARs were also observed in osteo-
184 cytes embedded in the bone matrix. Importantly, both the pattern of AR distribution and the
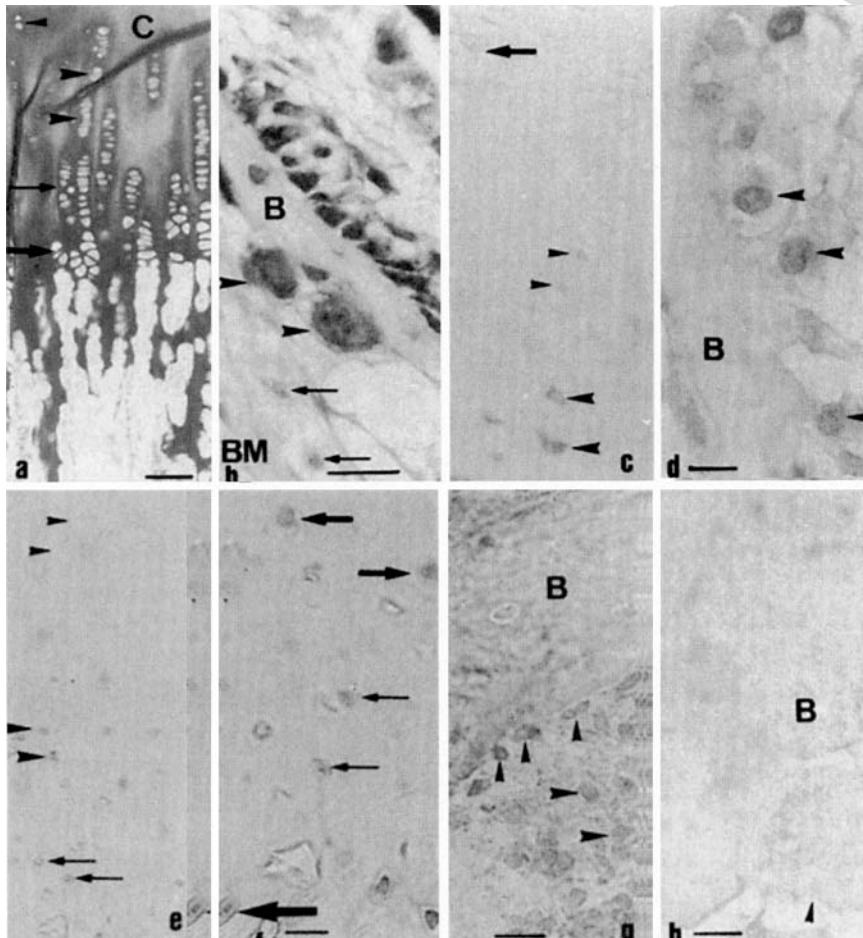
Fig. 2. The localization of AR in normal tibial growth plate and adult osteophytic human bone. (**a**) Morphologically, sections of the growth plate consist of areas of endochondral ossification with undifferentiated (*small arrow head*), proliferating (*large arrow heads*), mature (*small arrow*), and hypertrophic (*large arrow*) chondrocytes. Bar $= 80\,\mu$m. An inset of an area of the primary spongiosa is shown in (**b**). (**b**) Numerous osteoblasts (*small arrow heads*) and multinucleated osteoclasts (*large arrow heads*) on the bone surface. Mononuclear cells within the bone marrow are also present (*arrows*). Bar $= 60\,\mu$m. (**c**) In the growth plate, AR is predominantly expressed by hypertrophic chondrocytes (*large arrow heads*). Minimal expression is observed in the mature chondrocytes (*small arrow heads*). The receptors are rarely observed in the proliferating chondrocytes (*arrow*). (**d**) In the primary spongiosa, the AR is predominantly and highly expressed by osteoblasts at modeling sites (*arrow heads*). Bar $= 20\,\mu$m. (**e**) In the osteophytes, AR is also observed at sites of endochondral ossification in undifferentiated (*small arrow heads*), proliferating (*large arrow heads*), mature (*small arrows*), and hypertrophic-like (*large arrow*) chondrocytes. Bar $= 80\,\mu$m. (**f**) A higher magnification of (**e**) showing proliferating, mature, and hypertrophic-like chondrocytes (*large arrows*, *small arrows*, and *very large arrows*, respectively). Bar $= 40\,\mu$m. (**g**) At sites of bone remodeling, the receptors are highly expressed in the osteoblasts (*small arrow heads*) and also in mononuclear cells in the bone marrow (*large arrow heads*). Bar $= 40\,\mu$m. (**h**) AR is not detected in osteoclasts (*small arrow heads*). Bar $= 40\,\mu$m. B, Bone; C, cartilage; BM, bone marrow. Adapted from Abu et al. *(10)* and used with permission.

level of expression were similar in males and in females. In addition, expression of the AR has been characterized in cultured osteoblastic cell populations isolated from bone biopsy specimens determined at both the mRNA level and by binding analysis (35). Expression varied according to the skeletal site of origin and age of the donor of the cultured osteoblastic cells: AR expression was higher at cortical and intramembranous bone sites and lower in trabecular bone. This distribution pattern correlates with androgen responsiveness in the bone compartment. AR expression was highest in osteoblastic cultures generated from young adults and somewhat lower in samples from either prepubertal or senescent bone. Again, no differences were found between male and female samples, suggesting that differences in receptor number per se do not underlie development of a sexually dimorphic skeleton. Interestingly, ARs are also expressed in bone marrow stromal (55) and mesenchymal precursor cells (56), pluripotent cells that can differentiate into muscle, bone, and fat. Androgen action may modulate precursor differentiation toward the osteoblast and/or myoblast lineage, while inhibiting differentiation toward the adipocyte lineage (57). These effects on stromal differentiation could underlie some of the well-described consequences of androgen administration on body composition including increased muscle mass (58). To date, it has not been established how significant the contribution is, of the increased muscle mass associated with androgen administration, to positively influence bone quality. Bone marrow stomal cells are also responsive to sex steroids during the regulation of osteoclastogenesis.

Because androgens are so important in bone development at the time of puberty, it is not surprising that ARs are also present in epiphyseal chondrocytes (10,59). Noble and coworkers (54) described AR expression mainly in the narrow zone of proliferating chondrocytes in the growth plate, with reduced expression in hypertrophied cells. The expression of ARs in such a wide variety of cell types known to be important for bone modeling during development, and remodeling in the adult, provides strong evidence for direct actions of androgens in bone and cartilage tissue. These results also presage the complexity of androgen effects on developing bone tissue.

Potential modulation of osteoclast action by androgen is suggested by reports of AR expression in the osteoclast (60). Androgen treatment reduces bone resorption of isolated osteoclasts (61), inhibits osteoclast formation (62) including formation stimulated by parathyroid hormone (PTH) (63), and may play a direct role regulating aspects of osteoclast activity based on results in AR null mice (64). Indirect effects of androgen to modulate osteoclasts via osteoblasts are indicated by the increase in osteoprotegerin (OPG) levels following testosterone treatment in osteoblasts (65) and increased OPG serum concentrations in skeletally targeted AR-transgenic male mice (66). In addition, DHEA treatment has been shown to increase the OPG/RANKL ratio in osteoblastic cells and to inhibit osteoclast activity in coculture (67). Although androgen may be a less significant determinant of bone resorption in vivo than estrogen (68,69), this remains controversial (70).

Regulation of Androgen Receptor Expression

The regulation of AR expression in osteoblasts is incompletely understood. Homologous regulation of AR mRNA by androgen has been described that is tissue specific; upregulation by androgen exposure is seen in a variety of mesenchymal cells including osteoblasts (71–74), whereas in prostate and smooth muscle tissue, downregulation is observed after androgen exposure (73,75) (Fig. 3). The androgen-mediated up-regulation

Fig. 3. Dichotomous regulation of AR mRNA levels in osteoblast-like and prostatic carcinoma cell lines after exposure to androgen. (**A**) Time course of changes in AR mRNA abundance after DHT exposure in human SaOS-2 osteoblastic cells and human LNCaP prostatic carcinoma cells. To determine the effect of androgen exposure on hAR mRNA abundance, confluent cultures of either osteoblast-like cells (SaOS-2) or prostatic carcinoma cells (LNCaP) were treated with 10^{-8} M DHT for 0, 24, 48, or 72 h. Total RNA was then isolated and subjected to RNase protection analysis with 50 μg total cellular RNA from SaOS-2 osteoblastic cells and 10 μg total RNA from LNCaP cultures. (**B**) Densitometric analysis of AR mRNA steady state levels. The AR mRNA to β-actin ratio is expressed as the mean ± SE compared to the control value from three to five independent assessments. Adapted from Wiren et al. *(73)* and used with permission.

observed in osteoblasts occurs, at least in part, through changes in AR gene transcription *(73,74)*. No effect, or even inhibition, of AR mRNA by androgen exposure in other osteoblastic models has also been described *(35,76)*. Interestingly, a novel property of the AR is that binding of androgen increases AR protein levels that has been shown in osteoblastic cells as well *(74)*. This property distinguishes AR from most other steroid receptor molecules that are downregulated by ligand binding. The elevated AR protein levels may be a consequence of increased stability mediated by androgen binding, resulting from N-terminal and C-terminal interactions *(77)*, but the stability of AR protein in osteoblastic cells has not been determined to date. The mechanism(s) that underlie tissue specificity in autologous AR regulation, and the possible biological significance, is not

Fig. 4. Expression analyses of ERα, ERβ, and AR during in vitro differentiation in normal rat osteoblastic (rOB) cultures. (**A**) Normal rOB cells were cultured for the indicated number of days during proliferation, matrix maturation, mineralization, and postmineralization stages. Total RNA was isolated and subjected to relative RT-PCR analysis using primers specific for rat ERα, ERβ, and AR or rat GAPDH. Reverse

369   yet understood. It is possible that AR up-regulation by androgen in bone may result in
370   an enhancement of androgen responsiveness at times when androgen levels are rising or
371   elevated.

372   Quantitative determination of the level of receptor expression during osteoblast dif-
373   ferentiation is difficult to achieve in bone slices. However, analysis of AR, ERα and
374   ERβ mRNA, and protein expression during osteoblast differentiation in vitro demonstrates
375   that each receptor displays distinct differentiation-stage expression patterns in osteoblasts
376   (Fig. 4) *(78)*. The levels of AR expression increase throughout osteoblast differentiation
377   with the highest AR levels seen in mature osteoblast/osteocytic cultures. These results
378   suggest that an important compartment for androgen action may be mature, mineralizing
379   osteoblasts, and indicate that osteoblast differentiation and steroid receptor regulation are
380   intimately associated. Given that the osteocyte is the most abundant cell type in bone, and
381   a likely mediator of focal bone deposition and response to mechanical strain *(79)*, it is not
382   surprising that androgens may also augment the osteo-anabolic effects of mechanical strain
383   in osteoblasts *(80)*.

EFFECTS OF ANDROGENS ON OSTEOBLASTIC CELLS

387   Evidence suggests that androgens act directly on the osteoblast and there are reports,
388   some in clonal osteoblastic cell lines, of modulatory effects of gonadal androgen treatment
389   on proliferation, differentiation, matrix production, and mineral accumulation *(81)*. Not
390   surprisingly, androgen has been shown to influence bone cells in a complex fashion.

Androgens and Osteoblast Proliferation

394   As an example, the effect of androgen on osteoblast proliferation has been shown to be
395   biphasic in nature, with enhancement following short or transient treatment but significant
396   inhibition following longer treatment. As a case in point, Kasperk et al. *(82,83)* demon-
397   strated in osteoblast-like cells in primary culture (murine, passaged human) that a variety
398   of androgens in serum-free medium increase DNA synthesis ($[^3H]$thymidine incorporation)
399   and cell counts. Testosterone and nonaromatizable androgens (DHT and fluoxymesterone)
400   were nearly equally effective regulators. Yet the same group *(84)* reported that prolonged
401   DHT treatment inhibited normal human osteoblastic cell proliferation (cell counts) in cul-
402   tures pretreated with DHT. Hofbauer et al. *(85)* examined the effect of DHT exposure on
403   proliferation in hFOB/AR-6, an immortalized human osteoblastic cell line stably trans-
404   fected with an AR expression construct (with ∼4000 receptors/cell). In this line, DHT

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408   **Fig. 4.** (continued) transcription was conducted with PCR carried out for 40 cycles for the steroid recep-
409   tors, with parallel reactions performed using GAPDH primers for 25 cycles (all in the linear range).
410   Bands for rat ERα at the predicted 240 bp, rat ERβ at 262 bp, rat AR at 276 bp, and GAPDH at
411   609 bp are shown. (**B**) Analyses of ERα, ERβ, and AR mRNA relative abundance. Semi-quantitative
412   analysis of mRNA steady-state expression by relative RT-PCR was performed after scanning the negative
413   image of the photographed gels. Data are expressed in arbitrary units as the ratio of receptor abundance
414   to GAPDH expression, then normalized to expression values at day 4 in pre-confluent cultures. Data
       represent mean ± SEM. Adapted from Wiren et al. *(78)* and used with permission.

415 treatment inhibited cell proliferation by 20–35%. Consistent with stimulation, Somjen
416 et al. *(86)* have demonstrated increased creatine kinase-specific activity in male osteoblastic
417 cells after exposure to DHT for 24 h. Although these various studies employed differ-
418 ent model systems and culture conditions, it appears that exposure time is an important
419 variable. Clear time dependence for the response to androgen has been shown by Wiren
420 et al. *(87)*, where osteoblast proliferation was stimulated at early treatment times, but with
421 more prolonged DHT treatment osteoblast viability decreased (Fig. 5). This result was
422 AR dependent (inhibitable by coincubation with flutamide) and was observed in both nor-
423 mal rat calvarial osteoblasts and in AR stably transfected MC-3T3 cells. In mechanistic
424 terms, reduced viability was associated with overall reduction in mitogen-activated (MAP)
425 kinase signaling and with inhibition of *elk-1* gene expression, protein abundance, and extent
426 of phosphorylation. The inhibition of MAP kinase activity after chronic androgen treat-
427 ment again contrasts with stimulation of MAP kinase signaling and AP-1 transactivation
428 observed with brief androgen exposure *(87)* that may be mediated through non-genomic
429 mechanisms *(47,88,89)*.

Fig. 5. Complex effect of androgen on DNA accumulation in osteoblastic cultures. Kinetics of DHT response in proliferating colAR-MC3T3 cultures measured with colorimetric [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay. Cultures of stably transfected colAR-MC3T3 continuously with 10^{-8} M DHT for 2 days led to increased MTT accumulation, but longer treatment for 3 or 5 days resulted in inhibition. Data are mean $\pm$ SEM of six to eight dishes with six wells/dish. $*p < 0.05$; $**p < 0.01$ (vs. control). Adapted from Wiren et al. *(87)* and used with permission.

Androgens and Osteoblast Apoptosis

As a component of control of osteoblast survival, it is also important to consider the process of programmed cell death or apoptosis *(90)*. In particular, as the osteoblast population differentiates in vitro, the mature bone cell phenotype undergoes apoptosis *(91)*. With respect to the effects of androgen exposure, chronic DHT treatment has been shown to result in enhanced osteoblast apoptosis in both proliferating osteoblastic (at day 5) and mature osteocytic cultures (day 29) *(92)*. In this report, inhibition observed with DHT treatment was opposite to inhibitory effects on apoptosis seen with E_2 treatment (Fig. 6). An androgen-mediated increase in the Bax/Bcl-2 ratio was also observed, predominantly through inhibition of Bcl-2, and was dependent on functional AR. Overexpression of *bcl-2* or RNAi knockdown of *bax* abrogated the effects of DHT, indicating that increased Bax/Bcl-2 was necessary and sufficient for androgen-enhanced apoptosis. The increase in the Bax/Bcl-2 ratio was at least in part a consequence of reductions in Bcl-2 phosphorylation and protein stability, consistent with inhibition of MAP kinase pathway activation after DHT treatment as noted above. In vivo analysis of calvaria in AR-transgenic male mice demonstrated enhanced TUNEL staining in both osteoblasts and osteocytes and was observed even in areas of new bone growth *(92)*. This may not be surprising, given an association between new bone growth and apoptosis *(93)*, as has been observed in other remodeling tissues and/or associated with development and tissue homeostasis *(94)*. Apoptotic cell death could thus be important in making room for new bone formation and matrix deposition, which may have clinical significance by influencing bone homeostasis

Fig. 6. Characterization of osteoblast apoptosis: results of androgen and estrogen treatment during proliferation (day 5) and during differentiation into mature osteoblast/osteocytes cultures (day 29). Apoptosis was assessed at day 5 or 29 after continuous DHT and E_2 treatment (both at 10^{-8} M). Apoptosis was induced by etoposide treatment in proliferating cultures and by serum starvation for 48 h in confluent cultures before isolation, replaced with 0.1% BSA. (**A**) Analysis of apoptosis after evaluating DNA fragmentation by cytoplasmic nucleosome enrichment at day 5. The data are expressed as mean $\pm$ SEM ($n = 6$) from two independent experiments. $**p < 0.01$, $***p < 0.001$ (vs. control). (**B**) Analysis of apoptosis by cytoplasmic nucleosome enrichment analysis at day 29. The data are expressed as mean $\pm$ SEM ($n = 6$) from two independent experiments. $**p < 0.01$ vs. control. Adapted from Wiren et al. *(92)* and used with permission.

507 and bone mineral density *(95)*. Thus, mounting evidence suggests that chronic androgen
508 treatment does not increase osteoblast number or viability in the mature bone compart-
509 ment. It is interesting to speculate that the inhibitory action of androgens in osteoblasts at
510 the endosteal surface is important for the relative maintenance of cortical width (which is
511 similar between males and females), given the strong stimulation at the periosteal surface,
512 such that the skeleton does not become excessively large and/or heavy during development.

Effects of Androgens on the Differentiation of Osteoblastic Cells

516 Osteoblast differentiation is often characterized by changes in alkaline phosphatase
517 activity and/or alterations in the expression of important extracellular matrix proteins,
518 such as type I collagen, osteocalcin, and osteonectin. Enhanced osteoblast differentiation,
519 as measured by increased matrix production, has been shown to result from androgen
520 exposure. Androgen treatment in both normal osteoblasts and transformed clonal human
521 osteoblastic cells (TE-89) appears to increase the proportion of cells expressing alkaline
522 phosphatase activity, thus representing a shift toward a more differentiated phenotype *(82)*.
523 Kasperk et al. subsequently reported dose-dependent increases in alkaline phosphatase
524 activity in both high and low-alkaline phosphatase subclones of SaOS2 cells *(96)* and
525 human osteoblastic cells *(84)*. However, there are also reports in a variety of model systems
526 of androgens either inhibiting *(85)* or having no effect on alkaline phosphatase activity
527 *(71,97)*, which may reflect both the complexity and the dynamics of osteoblastic differ-
528 entiation. There are also reports of androgen-mediated increases in type I α-1 collagen
529 protein and mRNA levels *(37,96–98)* in certain circumstances and increased osteocal-
530 cin mRNA or protein secretion *(84,98)*. Consistent with increased collagen production,
531 androgen treatment has also been shown to stimulate mineral accumulation in a time and
532 dose-dependent manner *(71,84,99)*. However, transgenic mice with targeted overexpression
533 of AR in the osteoblast lineage showed decreased levels of most bone markers in vivo in
534 total RNA extracts derived from long bone samples, including decreased collagen, osterix,
535 and osteocalcin gene expression *(66)*. These results suggest that, under certain conditions,
536 androgens may enhance osteoblast differentiation and could thus play an important role
537 in the regulation of bone matrix production and/or organization. On the other hand, many
538 positive anabolic effects of androgen may be limited to distinct osteoblastic populations,
539 for example, in the periosteal compartment *(2,66)*.

Interaction with Other Factors to Modulate Bone Formation and Resorption

543 The effects of androgens on osteoblast activity must certainly also be considered in the
544 context of the very complex endocrine, paracrine, and autocrine milieu in the bone microen-
545 vironment. Systemic and/or local factors can act in concert, or can antagonize, to influence
546 bone cell function. This has been well described with regard to modulation of the effects of
547 estrogen on bone (see, for example, *(100–102)*). Androgens have also been shown to reg-
548 ulate well-known modulators of osteoblast proliferation or function. The most extensively
549 characterized growth factor influenced by androgen exposure is transforming growth factor-
550 β (TGF-β). TGF-β is stored in bone (the largest reservoir for TGF-β) in a latent form and has
551 been shown to be a mitogen for osteoblasts. Androgen treatment can increase TGF-β activ-
552 ity in osteoblastic cultures: the expression of some TGF-β mRNA transcripts (apparently

553 TGF-β2) was increased but no effect on TGF-β1 mRNA abundance was observed *(41,83)*,
554 but also see *(103)*. At the protein level, specific immunoprecipitation analysis reveals DHT-
555 mediated increases in TGF-β activity to be predominantly TGF-β2 *(41,84)*. DHT has also
556 been shown to inhibit both TGF-β gene expression and TGF-β-induced early gene expres-
557 sion that correlates with growth inhibition in this cell line *(85)*. The TGF-β-induced early
558 gene has been shown to be a transcription factor that may mediate some TGF-β effects
559 *(104)*. These results are consistent with the notion that TGF-β may mediate androgen
560 effects on osteoblast proliferation. On the other hand, TGF-β1 mRNA levels are increased
561 by androgen treatment in human clonal osteoblastic cells (TE-89), under conditions where
562 osteoblast proliferation is slowed *(37)*. Thus, the specific TGF-β isoform may determine
563 osteoblast responses. It is interesting to note that in vivo, orchiectomy (ORX) drastically
564 reduces bone content of TGF-β levels, and testosterone replacement prevents this reduc-
565 tion *(105)*. These data support the findings that androgens influence cellular expression of
566 TGF-β and suggest that the bone loss associated with castration is related to a reduction in
567 growth factor abundance induced by androgen deficiency.

568 Other growth factor systems may also be influenced by androgens. Conditioned
569 media from DHT-treated normal osteoblast cultures are mitogenic, and DHT pretreatment
570 increases the mitogenic response to fibroblast growth factor and to insulin-like growth
571 factor II (IGF-II) *(83)*. In part, this may be due to slight increases in IGF-II binding in
572 DHT-treated cells *(83)*, as IGF-I and IGF-II levels in osteoblast-conditioned media are not
573 affected by androgen *(83,106)*. Although most studies have not found regulation of IGF-I
574 or IGF-II abundance by androgen exposure *(33,83,106)*, there is a report that IGF-I mRNA
575 levels are significantly up-regulated by DHT *(107)*. Androgens may also modulate expres-
576 sion of components of the AP-1 transcription factor *(41)* or AP-1 transcriptional activation
577 *(87)*. Thus, androgens may modulate osteoblast differentiation via a mechanism whereby
578 growth factors or other mediators of differentiation are regulated by androgen exposure.

579 Androgens may modulate responses to other important osteotropic hormones/regulators.
580 Testosterone and DHT specifically inhibit the cAMP response elicited by PTH or parathy-
581 roid hormone-related protein (PTHrP) in the human clonal osteoblast-like cell line SaOS-2,
582 while the inactive or weakly active androgen 17α-epitestosterone had no effect, via an effect
583 on effector G_s-adenylyl cyclase *(108–110)*. The production of prostaglandin E_2 (PGE$_2$),
584 another important regulator of bone metabolism, is also affected by androgens. Pilbeam and
585 Raisz showed that androgens (both DHT and testosterone) were potent inhibitors of both
586 parathyroid hormone and interleukin-1-stimulated PGE$_2$ production in cultured neonatal
587 mouse calvaria *(111)*. The effects of androgens on parathyroid hormone action and PGE$_2$
588 production suggest that androgens could act to modulate (reduce) bone turnover in response
589 to these agents.

590 Finally, both androgen *(112)* and estrogen *(101,113)* can inhibit production of
591 interleukin-6 by osteoblastic cells (but see *(114)*). In stromal cells of the bone mar-
592 row, androgens have been shown to have potent inhibitory effects on the production
593 of interleukin-6 and the subsequent stimulation of osteoclastogenesis by marrow osteo-
594 clast precursors *(115)*. Interestingly, adrenal androgens (androstenediol, androstenedione,
595 DHEA) have similar inhibitory activities on interleukin-6 gene expression and protein pro-
596 duction by stromal cells *(115)*. Moreover, androgens inhibit the expression of the genes
597 encoding the two subunits of the IL-6 receptor (gp80 and gp130) in the murine bone mar-
598 row, another mechanism which may blunt the effects of this osteoclastogenic cytokine in

intact animals *(116)*. In these aspects, the effects of androgens seem to be very similar to those of estrogen, which may also inhibit osteoclastogenesis via mechanisms that involve interleukin-6 inhibition and/or OPG/RANKL ratio changes.

METABOLISM OF ANDROGENS IN BONE

Sex steroids, ultimately derived from cholesterol, are synthesized predominantly in gonadal tissue, the adrenal gland, and placenta as a consequence of enzymatic conversions. After peripheral metabolism, androgenic activity is represented in a variety of steroid molecules that include testosterone (Fig. 7). There is evidence in a range of tissues that the eventual cellular effects of testosterone may not be the result (or not only the result) of direct action of testosterone, but may also reflect the effects of sex steroid metabolites formed as a consequence of local enzyme activities.

The most important testosterone metabolites in bone are 5α-DHT (the result of 5α reduction of testosterone) and estradiol (formed by the aromatization of testosterone). Testosterone and DHT are the major and most potent androgens, with androstenedione (the major circulating androgen in women) and DHEA as immediate androgen precursors that exhibit weak androgen activity *(39)*. In men, the most abundant circulating androgen

Pathways of androgen metabolism

Fig. 7. Principle conversions and major enzyme activities involved in androgen synthesis and metabolism. Steroid hormone synthesis involves metabolism of cholesterol, with dehydrogenation of pregnenolone producing progesterone that can serve as a precursor for the other gonadal steroid hormones. DHEA, dehydroepiandrosterone; CYP11A, cytochrome P450 cholesterol side chain cleavage enzyme; CYP17, cytochrome P450 17α hydroxylase/17,20 lyase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; CYP19, aromatase cytochrome P450.

645 metabolite is testosterone while concentrations of other weaker androgens-like androstene-
646 dione and DHEA-sulfate are similar between males and females. Downstream metabolites
647 of DHT and androstenedione are inactive at the AR and include 5α-androstane-3α or
648 3β,17β-diol (3αβ-androstanediol), and 5α-androstanedione. Data suggest that aromatase
649 cytochrome P450 (the product of the CYP19 gene), 17β-hydroxysteroid dehydrogenase
650 (17β-HSD), and 5α-reductase activities are all present in bone tissue, at least to some mea-
651 surable extent in some compartments, but the biologic relevance of each remains somewhat
652 controversial.

5α-Reductase Activity in Osteoblasts

655 5α-reductase is an important activity with regard to androgen metabolism in general,
656 since testosterone is converted to the more potent androgen metabolite DHT via 5α-
657 reductase action *(117)*. 5α-Reductase activity was first described in crushed rat mandibular
658 bone *(118)* with similar findings reported in crushed human spongiosa *(119)*. Two differ-
659 ent 5α-reductase genes encode type 1 and type 2 isozymes in many mammalian species
660 *(120)*; human osteoblastic cells express the type 1 isozyme *(121)*. Essentially the same
661 metabolic activities were reported in experiments with human epiphyseal cartilage and
662 chondrocytes *(122)*. In general, the K_m values for bone 5α-reductase activity are similar
663 to those in other androgen responsive tissues *(33,119)*. However, the cellular populations
664 in many of these studies were mixed and hence the specific cell type responsible for the
665 activity is unknown. Interestingly, Turner et al. *(123)* found that periosteal cells do not have
666 detectable 5α-reductase activity, raising the possibilities that the enzyme may be functional
667 in only selected skeletal compartments and that testosterone may be the active androgen
668 metabolite at this clinically important site.

669 From a clinical perspective, the general importance of this enzymatic pathway is uncer-
670 tain, as patients with 5α-reductase type 2 deficiency have normal bone mineral density *(124)*
671 and Bruch et al. *(117)* found no significant correlation between enzyme activities and bone
672 volume. In mutant null mice lacking 5α-reductase type 1 (mice express very little type 2
673 isozyme), the effect on the skeleton has not been analyzed due to midgestational fetal death
674 as a consequence of estrogen excess *(125)*. Analysis of the importance of 5α-reductase
675 activity has been approached with the use of finasteride (an inhibitor of 5α-reductase activ-
676 ity); treatment of male animals does not recapitulate the effects of castration *(126)*, strongly
677 suggesting that reduction of testosterone to DHT by 5α-reductase is not the major determi-
678 nant in the effects of gonadal hormones on bone. Consistent with this finding, testosterone
679 therapy in hypogonadal older men, either when administered alone or when combined with
680 finasteride, increases bone mineral density, again suggesting that DHT is not essential for
681 the beneficial effects of testosterone on bone *(127)*. Thus, the available clinical data remain
682 uncertain, and the impact of this enzyme, which isozyme may be involved, whether it is
683 uniformly present in all cell types involved in bone modeling/remodeling, or whether local
684 activity is important at all remain unresolved issues.

Aromatization of Testosterone in Bone

687 Another important enzymatic arm of testosterone metabolism involves the biosynthe-
688 sis of estrogens from androgen precursors, catalyzed by aromatase. Of note, this enzyme is
689 well known to be both expressed and regulated in a very pronounced tissue-specific manner
690 *(128)* and also demonstrates species differences, given the low levels in mice. Modest levels

691 of aromatase activity have been reported in bone from mixed cell populations derived from
692 both sexes *(129–131)* and from osteoblastic cell lines *(33,132,133)*. Aromatase expression
693 in intact bone has also been documented by in situ hybridization and immunohistochemical
694 analysis *(131)*. Aromatase mRNA is expressed predominantly in lining cells, chondrocytes,
695 and some adipocytes; however, there is no detectable expression in osteoclasts, or in cor-
696 tical bone in mice *(66)*. At least in vertebral bone, the mesenchymal distal promoter I.4 is
697 predominantly utilized *(134)*. The enzyme kinetics in bone cells seem to be similar to those
698 in other tissues, although the V_{max} may be increased by glucocorticoids *(133)*. Whether
699 the level of aromatase activity in bone is high enough to produce physiologically relevant
700 concentrations of steroids remains an open question; nevertheless in males only 15% of cir-
701 culating estrogen is produced in the testes, with the remaining 85% produced by peripheral
702 metabolism that could include bone as one site of conversion *(135)*.

703 Aromatase catalyzes the metabolism of adrenal and testicular C19 androgens
704 (androstenedione and testosterone) to C18 estrogens (estrone and estradiol), thus producing
705 the potent estrogen estradiol (E2) from testosterone, and the weaker estrogen estrone (E1)
706 from its adrenal precursors androstenedione and DHEA *(129)*. Typically in the circulation,
707 E2 will make up to 40% of total estrogen, E1 will make up an additional 40%, with estriol
708 (E3) comprising the remaining 20% of total estrogen *(136)*. In addition to aromatase itself,
709 osteoblasts contain enzymes that are able to inter-convert estradiol and estrone (17β-HSD)
710 and to hydrolyze estrone sulfate, the most abundant estrogen in the circulation, to estrone
711 (steroid sulfatase) *(132,137)*. Nawata et al. *(129)* have reported that dexamethasone and
712 $1\alpha,25(OH)_2D_3$ synergistically enhance aromatase activity and aromatase mRNA expres-
713 sion in human osteoblast-like cells. In addition, both leptin and $1\alpha,25(OH)_2D_3$ treatment
714 increased aromatase activity in human mesenchymal stem cells during osteogenesis, but
715 not during adipogenesis *(138)*. Additional studies are needed to better define expression,
716 given the potential importance of the enzyme, and its regulation by a variety of mechanisms
717 (including androgens and estrogens) in other tissues *(128,139)*.

718 The clinical impact of aromatase activity and an indication of the importance of conver-
719 sion of circulating androgen into estrogen are shown in reports of women and men with
720 aromatase deficiencies, who present with a skeletal phenotype *(140)*. Interestingly, natural
721 mutation is remarkably rare with only seven males and six females reported to date. The
722 presentation of men with aromatase deficiency is very similar to that of a man with estrogen
723 receptor-α (ERα) deficiency *(141)*, namely an obvious delay in bone age, lack of epiphy-
724 seal closure, and tall stature with high bone turnover and osteopenia *(135)*, suggesting that
725 aromatase (and likely estrogen action) has a substantial role to play during skeletal devel-
726 opment in the male. In addition, estrogen therapy of males with aromatase deficiency has
727 been associated with an increase in bone mass *(135)*, particularly in the growing skeleton
728 *(142)*. Inhibition of aromatization pharmacologically with nonsteroidal inhibitors (such as
729 vorozole or letrozole) results in modest decreases in bone mineral density and changes in
730 skeletal modeling in young growing orchidectomized males *(143)*, and less dramatically so
731 in boys with constitutional delay of puberty treated for 1 year *(144)*, suggesting short-term
732 treatment during growth has limited negative consequences in males. Inhibition of aroma-
733 tization in older orchidectomized males resembles castration with similar increases in bone
734 resorption and bone loss, suggesting that aromatase activity likely plays a role in skeletal
735 maintenance in males *(145)*. These studies herald the importance of aromatase activity (and
736 estrogen) in the mediation of some androgen action in bone in both males and females. The

737 finding of these enzymes in bone clearly raises the difficult issue of the origin of androgenic
738 effects in the skeleton; do they arise solely from direct androgen effects (as is suggested by
739 the actions of nonaromatizable androgens such as DHT) or also from the local or other site
740 production of estrogenic intermediates? The results described above would seem to indicate
741 that both steroids appear to be important to both male and female skeletal health.

743 ### *17β-Hydroxysteroid Dehydrogenase Activity in Osteoblasts*

744 The 17β-HSDs (most of which are dehydrogenase-reductases, except type 5 that is an
745 aldo–keto reductase) have been shown to catalyze either the last step of sex steroid synthesis
746 or the first step of their degradation (to produce weak or potent sex steroids via oxida-
747 tion or reduction, respectively) and can thus also play a critical role in peripheral steroid
748 metabolism. The oxidative pathway forms 17-ketosteroids, while the reductive pathway
749 forms 17β-hydroxysteroids. The enzyme reversibly catalyzes the formation of androstene-
750 diol (an estrogen) from DHEA, in addition to the biosynthesis of estradiol from estrone,
751 the synthesis of testosterone from androstenedione, and the production of DHT from 5α-
752 androstanedione all via the reductive activity of 17β-HSD. Of the 13 enzyme isotypes of
753 17β-HSD activity *(136)*, types 1–4 have been demonstrated in human osteoblastic cells
754 *(146)*.

755 The administration of testosterone can stimulate bone formation and inhibit bone
756 resorption, likely through multiple mechanisms that involve both androgen and estrogen
757 receptor-mediated processes. However, there is substantial evidence that some, if in fact
758 not most, of the biologic actions of androgens in the skeleton are mediated by AR. Both
759 in vivo and in vitro systems reveal the effects of the nonaromatizable androgen DHT to
760 be essentially the same as those of testosterone (vida infra). In addition, blockade of the
761 AR with the receptor antagonist flutamide results in osteopenia as a result of reduced bone
762 formation *(5)*. In addition, complete androgen insensitivity results in a significant decrease
763 in bone mineral density in spine and hip sites *(124)* even in the setting of strong compliance
764 with estrogen treatment *(147)*. These reports clearly indicate that androgens, independent of
765 estrogenic metabolites, have primary effects on osteoblast function. However, the clinical
766 reports of subjects with aromatase deficiency also highlight the relevance of metabolism of
767 androgen to bio-potent estrogens at least in the circulation, to influence bone development
768 and/or maintenance. It thus seems likely that further elucidation of the regulation of steroid
769 metabolism, and the potential mechanisms by which androgenic and estrogenic effects are
770 coordinated, will have physiological, pathophysiological, and therapeutic implications.

772 ### *Drugs with Androgenic Activity*

773 In addition to the endogenous steroid metabolites highlighted in Fig. 7, there are also a
774 variety of drugs with androgenic activity. These include anabolic steroids, such as nonarom-
775 atizable oxandrolone, that bind and activate AR (albeit with lower affinity than testosterone
776 *(148)*), and a class of drugs under extensive development referred to as SARMs that demon-
777 strate tissue-specific agonist or antagonist activities with respect to AR transactivation
778 *(149)*. These orally active nonsteroidal nonaromatizable SARMS are being developed to
779 target androgen action in bone, muscle, fat and to influence libido but to not exacerbate
780 prostate growth, hirsutism, and acne. Several have recently been identified with beneficial
781 effects on bone mass *(150–152)*, and provide a new alternative to androgen replacement
782 therapy.

GENDER SPECIFICITY IN THE ACTIONS OF SEX STEROIDS

Although controversial, there may be gender-specific responses in osteoblastic cells to sex steroids. In most mammals, there is a marked gender difference in morphology that results in a sexually dimorphic skeleton. The mechanisms responsible for these differences are necessarily complex, and presumably involve both androgenic and estrogenic actions on the skeleton. It is becoming increasingly clear that estrogens are particularly important for the regulation of epiphyseal function and act to reduce the rate of longitudinal growth via influences on chondrocyte proliferation and function, as well as on the timing of epiphyseal closure *(153)*. Androgens, on the other hand, appear to have many opposite effects to estrogen on the skeleton. For example, androgens tend to promote long bone growth, chondrocyte maturation, and metaphyseal ossification, opposite to effects of estrogen. Another notable example is the effect of AR activation in cortical bone in males, which can stimulate bone formation at the periosteal surface but inhibit formation at the endosteum *(66)*. Thus, the most dramatic effect of androgens is on bone size, in particular cortical thickness *(154)*. This difference of course has important biomechanical implications, with thicker bones being stronger bones *(155)*. Furthermore, the response of the adult skeleton (to the same intervention) results in distinct responses in males and females. For example, in a model of disuse osteopenia, antiorthostatic suspension results in significant reduction in bone formation rate at the endosteal perimeter in males. In females, however, a decrease in bone formation rate occurred along the periosteal perimeter *(156)*. Gender-specific responses in vivo and in vitro (for example, see *(86)*), and the mechanism(s) that underlie such responses in bone cells, may thus have significant implications in treatment options for metabolic bone disease.

CONCLUSION

Thus, the effects of androgens on bone health are both complex and pervasive. Androgens influence skeletal modeling and remolding by multiple mechanisms through effects on osteoblasts, osteoclasts, and even perhaps an influence on the differentiation of pluripotent stem cells toward distinct lineages. The specific effects of androgen on bone cells are mediated directly through an AR-signaling pathway, but there are also indirect contributions to overall skeletal health through aromatization and ER signaling. The effects of androgens are particularly dramatic during growth in boys, particularly at the periosteum, but almost certainly play an important role during this period in girls as well. Throughout the rest of life, androgens affect skeletal function and maintenance in both sexes. Nevertheless, given this importance, relatively little has been done to unravel the mechanisms by which androgens influence the physiology and pathophysiology of bone, and there is still much to be learned about the roles of androgens at all levels. The interaction of androgens and estrogens and how their respective actions can be utilized for specific diagnostic and therapeutic benefit are important but unanswered issues. With an increase in the understanding of the nature of androgen effects will come greater opportunities to use their positive actions in the prevention and treatment of a wide variety of bone disorders.

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Chapter 16

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Androgens and Bone: Basic Aspects

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INTRODUCTION

s0010

p0010 Most research in gonadal steroid action on bone has focused on the effects of estrogen because of the obvious importance of the menopause in the development of osteoporosis. However, it is clear that androgens also have important effects on both skeletal development and the maintenance of bone mass and the mechanisms by which androgens affect skeletal homeostasis are becoming increasingly clear. Thus, it has been demonstrated that androgens:

o0010 1 influence growth plate maturation helping to determine longitudinal bone growth during development
o0020 2 mediate regulation of trabecular (cancellous) and cortical bone mass in a fashion distinct from estrogen, leading to the development of a sexually dimorphic skeleton
o0030 3 modulate peak bone mass acquisition
o0040 4 inhibit bone loss (for review see [1–3]).

p0060   A specific role for androgen in skeletal health is clear, at least in animal models. For example, in castrate animals, replacement with non-aromatizable androgens (e.g. 5α-dihydrotestosterone, DHT) yields beneficial effects that are clearly distinct from those observed with estrogen replacement [4,5]. In intact females, blockade of the androgen receptor (AR) with the specific AR antagonist hydroxyflutamide results in osteopenia [6]. Consistent with this finding, treatment of females with non-aromatizable androgen alone results in improvements in bone mineral density [7]. Finally, combination therapy with estrogen and androgen in postmenopausal women is more beneficial than either steroid alone [8–10]. Combined, these reports suggest the possibility of both distinct and overlapping actions of androgens and estrogens on the skeleton in both sexes. A growing awareness of the importance of the effects of androgen on bone and the potential to make use of this information for the treatment of bone disorders, has fuelled an increase in research.

ANDROGENS AND THE ROLE OF ANDROGEN METABOLISM IN BONE

s0020

p0070 All steroid hormones, including sex steroids, are derived from cholesterol. Sex steroids are synthesized as a consequence of enzymatic conversion, predominantly in gonadal tissue, the adrenal gland and placenta. After such peripheral metabolism, androgenic activity is represented in a variety of steroid molecules that include testosterone (Figure 25.1). There is evidence in a range of tissues that the eventual cellular effects of testosterone may not be the result (or not only the result) of direct action of testosterone, but may also reflect the effects of sex steroid metabolites formed as a consequence of local enzyme activities.

DHT

s0030

p0080 The most important testosterone metabolites active in bone are 5α-DHT (the result of 5α reduction of testosterone) and estradiol (formed by the aromatization of testosterone). Testosterone and DHT are the major and most potent androgens, with androstenedione (the major circulating androgen in women) and dehydroepiandrosterone (DHEA) as immediate androgen precursors that exhibit weak androgen activity [11]. In men, the most abundant circulating androgen metabolite is testosterone, while concentrations of other weaker androgens like androstenedione and DHEA-sulfate are similar between males and females. Downstream metabolites of androstenedione and DHT include 5α-androstanedione and 5α-androstane-3α or 3β,17β-diol (3α- or β-diol) respectively. Although these steroids are considered inactive at the AR, the DHT metabolites 3α-diol can function as an allosteric modulator to influence gamma-aminobutyric acid A (GABA$_A$) receptor function, while 3β-diol demonstrates estrogenic activity at estrogen receptor (ER)-β receptors [12]. 3α/β-Hydroxysteroid dehydrogenase activity has been shown in osteoblasts [13]. In sum, data suggest that aromatase cytochrome P450 (the
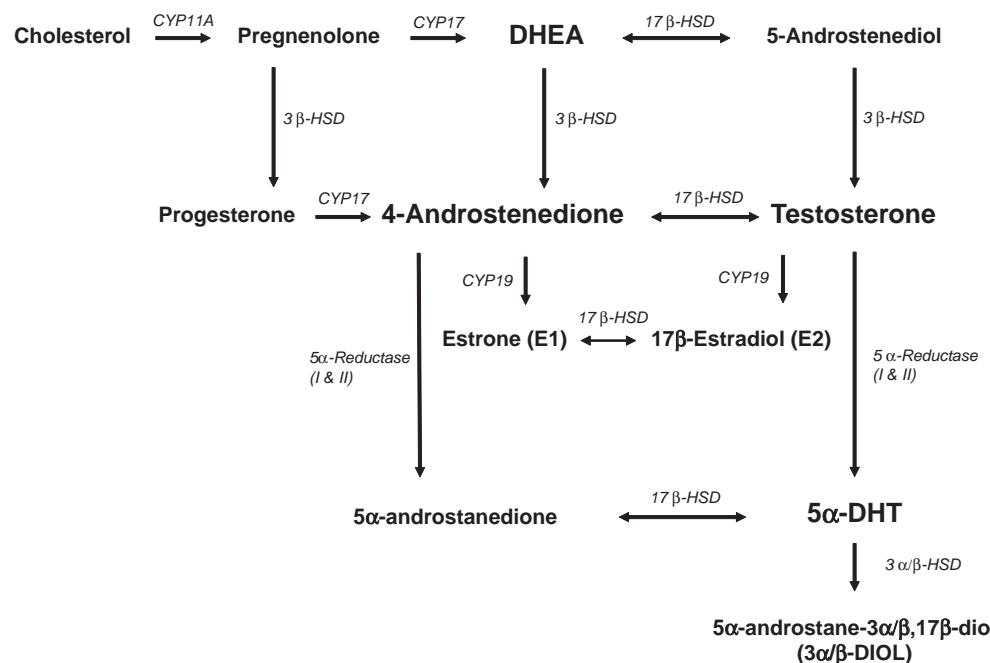
FIGURE 25.1   Principal conversions and major enzyme activities involved in androgen synthesis and metabolism. Steroid hormone synthesis involves metabolism of cholesterol, with dehydrogenation of pregnenolone producing progesterone that can serve as a precursor for the other gonadal steroid hormones. DHEA, dehydroepiandrosterone; CYP11A, cytochrome P450 cholesterol side chain cleavage enzyme; CYP17, cytochrome P450 17α hydroxylase/17,20 lyase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; CYP19, aromatase cytochrome P450.

product of the CYP19 gene), 17β-hydroxysteroid dehydrogenase (17β-HSD), 3α/β-HSD and 5α-reductase activities are all present in bone tissue, at least to some measurable extent in some compartments, but the biological relevance of each remains somewhat controversial.

p0090   5α-Reductase is an important activity with regard to androgen metabolism, since testosterone is converted to the more potent androgen metabolite DHT via 5α-reductase action. 5α-Reductase activity was first described in crushed rat mandibular bone [14] with similar findings reported in crushed human spongiosa [15]. Two different 5α-reductase genes encode type 1 and type 2 isozymes in many mammalian species; osteoblastic cells predominantly express the type 1 isozyme [13,16]. Essentially the same metabolic activities were reported in experiments with human epiphyseal cartilage and chondrocytes [17]. In general, the K_m values for bone 5α-reductase activity are similar to those in other androgen responsive tissues [15,18]. However, given that the cellular populations in most studies were mixed, the specific cell type responsible for the activity is unknown. Interestingly, Turner et al found that periosteal cells do not have detectable 5α-reductase activity [19], raising the possibilities that the enzyme may be functional in only selected skeletal compartments and that testosterone may be the active androgen metabolite at this clinically important site in bone as it is in muscle.

s0040   From a clinical perspective, the general importance

p0110   of this enzymatic activity is uncertain, as patients with 5α-reductase type 2 deficiency have normal bone mineral

density [20] and Bruch et al found no significant correlation between enzyme activities and bone volume [21]. In mutant null mice lacking 5α-reductase type 1 (mice express very little type 2 isozyme), the effect on the skeleton has not been analyzed due to mid-gestational fetal death as a consequence of estrogen excess [22]. In addition, analysis of the importance of 5α-reductase activity has been approached with the use of finasteride, an inhibitor of 5α-reductase activity (type 1 in humans; both types in rodents). Treatment of male animals does not recapitulate the effects of castration [23], strongly suggesting that reduction of testosterone to DHT is not a major determinant in the effects of gonadal hormones on bone. Consistent with this finding, testosterone therapy in hypogonadal older men, either when administered alone or when combined with finasteride, increases bone mineral density, again suggesting that DHT is not essential for the beneficial effects of testosterone on bone [24]. Combined, the available data do not support the general importance of this metabolic pathway. Thus, the impact of this enzyme, which isozyme may be involved, whether it is uniformly present in all cell types involved in bone modeling/remodeling, or whether local activity is important at all, remain unresolved issues.

Aromatase

p0100

A second primary enzymatic arm of testosterone metabolism involves the biosynthesis of estrogens from androgen

precursors, catalyzed by aromatase. Of note, this enzyme is well known to be both expressed and regulated in a very pronounced tissue-specific manner from a variety of promoters [25]. Aromatase also demonstrates pronounced species differences, given the low peripheral levels found in rodents [26], including in cortical bone in mice [27]. Modest levels of aromatase activity have been reported in human bone from mixed cell populations derived from both sexes [28–30] and from osteoblastic cell lines [31,32]. Aromatase expression in intact bone has also been documented by in situ hybridization and immunohistochemical analysis [30]. Aromatase mRNA is expressed predominantly in lining cells, chondrocytes and some adipocytes, however, there is no detectable expression in osteoclasts. The enzyme kinetics in bone cells appears similar to those in other tissues, although the V_{max} may be increased by glucocorticoids [32]. Whether the level of aromatase activity in bone is high enough to produce physiologically relevant concentrations of steroids remains an open question; nevertheless, in the male, only 15% of circulating estrogen is produced in the testes, with the remaining 85% produced by peripheral metabolism that, in addition to fat, could include bone as one site of conversion [33].

p0120   Aromatase catalyzes the metabolism of adrenal and testicular C19 androgens, such as androstenedione and testosterone, to the C18 estrogens estrone and estradiol. Aromatase thus produces the potent estrogen estradiol (E_2) from testosterone and the weaker estrogen estrone (E_1) from its adrenal precursors androstenedione and DHEA [28]. Typically in the circulation, E_2 will make up to 40% of total estrogen, E_1 will make up an additional 40%, with estriol (E_3) comprising the remaining 20% of total estrogen [34]. In addition to aromatase itself, osteoblasts contain enzymes that are able to interconvert estradiol and estrone (17β-HSD) and to hydrolyze estrone sulfate, the most abundant estrogen in the circulation, to estrone through steroid sulfatase. Synergistic enhancement of aromatase activity and aromatase mRNA expression is seen after treatment with dexamethasone and $1\alpha,25(OH)_2D_3$ [28], dexamethasone and forskolin [35] and dexamethasone and prostaglandin E(2) [36], in human osteoblast-like cells. In addition, both leptin and $1\alpha,25(OH)_2D_3$ treatment increased aromatase activity in human mesenchymal stem cells during osteogenesis, but not during adipogenesis [37]. Additional studies are needed better to define expression, given the potential importance of the enzyme, and its regulation by a variety of mechanisms (including androgens and estrogens) in other tissues.

The clinical impact of aromatase activity and an indication of the importance of conversion of circulating androgen into estrogen is shown in reports of women and men with aromatase deficiencies, who present with a skeletal phenotype [38]. Interestingly, natural mutation is remarkably rare with only seven males and ten females reported to date [39]. The presentation of men with aromatase deficiency

is very similar to that observed with a man with estrogen receptor-α (ERα) deficiency, namely an obvious delay in bone age, lack of epiphyseal closure and tall stature, with high bone turnover and osteopenia [33]. These findings suggest that aromatase (and likely estrogen action) has a substantial role to play during skeletal development in the male as well as the female. In addition, estrogen therapy of males with aromatase deficiency has been associated with an increase in bone mass and size [33,40], particularly in the growing skeleton. Pharmacological inhibition of aromatization using non-steroidal inhibitors, such as vorozole or letrozole, results in modest decreases in bone mineral density and changes in skeletal modeling in young growing orchidectomized males [41] and less dramatically so in boys with constitutional delay of puberty after treatment for one year [42], suggesting short-term treatment during growth has limited negative consequences in males. Inhibition of aromatization in older orchidectomized males resembles castration with similar increases in bone resorption and bone loss, suggesting that aromatase activity likely plays an important role in skeletal maintenance in males [43]. These studies speak to the importance of aromatase activity, and estrogen itself, in the mediation of some androgen action in bone in both males and females, although the importance in rodents may be reduced because of the low levels of aromatase expression in the periphery. The presence of these enzymes in the periphery, including to some extent in bone, clearly raises the difficult issue of the origin of androgenic effects in the skeleton; do they arise solely from direct androgen effects (as is suggested by the actions of non-aromatizable androgens such as DHT) and/or from the local or other site production of estrogenic intermediates? The results described above would indicate that both steroids appear to be important to both male and female skeletal health.

Androgen Precursors and Metabolites   s0050

The 17β-HSDs (most of which are dehyrogenase-reductases, p0140 except type 5 that is an aldo-keto reductase) have been shown either to catalyze the last step of sex steroid synthesis or the first step of their degradation (to produce weak or potent sex steroids via oxidation or reduction, respectively) and can thus also play a critical role in peripheral steroid metabolism. The oxidative pathway forms 17-ketosteroids while the reductive pathway forms 17β-hydroxysteroids. The enzyme reversibly catalyzes the formation of androstenediol (an estrogen) from DHEA, in addition to the biosynthesis of estradiol from estrone, the synthesis of p0130 testosterone from androstenedione and the production of DHT from 5α-androstanedione all via the reductive activity of 17β-HSD. Of the 13 enzyme isotypes of 17β-HSD activity, types 1–4 have been demonstrated in human osteoblastic cells [44]. Interestingly, ubiquitous overexpression of human 17β-HSD (HSD17B1) in female mice results in

masculinization, but specific characterization of the consequences in bone has not yet been reported [45]. Ubiquitous overexpression of human 17β-HSD type 2 delays skeletal development in male prepubertal mice, but has no effect on adults [46]. There are few data describing possible sex differences for the expression or activity for any of these metabolic enzymes in bone.

Synthetic Androgens

In addition to the endogenous steroid metabolites highlighted in Figure 25.1, there are also a variety of drugs with androgenic activity. These include the anabolic steroids, such as non-aromatizable nandrolone, that bind and activate AR (albeit with lower affinity than testosterone [47]). In addition, a class of drugs referred to as selective AR modulators (SARMs) are under extensive development and demonstrate tissue-specific agonist or antagonist activities with respect to AR transactivation. These orally active non-steroidal non-aromatizable SARMS are being developed to target positive androgen action in tissues such as bone, muscle, fat and to influence libido but, at the same time, not to exacerbate prostate growth, hirsutism and acne. Several have recently been identified with beneficial effects on bone mass in males and females, exclusively in a hypogonadal setting [48–53]. SARMS may thus provide a new alternative to androgen replacement therapy and potentially for age-related fragility.

Androgen versus Estrogen action

Thus, androgen effects in bone may occur through multiple complex mechanisms that involve testosterone, DHT, weaker androgens and androgen metabolites and the estrogens that are derived from the conversion of androgen precursors. Both androgen and estrogen receptor-mediated processes may mediate these effects in distinct skeletal compartments. Although estrogens exert a major influence on bone, there is compelling evidence that many of the biological actions of androgens in the skeleton are mediated via AR activation in males. Both in vivo and in vitro systems reveal the effects of the non-aromatizable androgen DHT to be essentially the same as those of testosterone (see below). In addition, blockade of the AR with the receptor antagonist flutamide results in osteopenia as a result of reduced bone formation [6]. In addition, complete androgen insensitivity results in a significant decrease in bone mineral density in spine and hip sites [20] even in the setting of strong compliance with estrogen treatment [54]. These reports clearly indicate that androgens, independent of estrogenic metabolites, have primary effects on osteoblast function. However, the clinical reports of subjects with aromatase deficiency also highlight the relevance of metabolism of androgen to bio-potent estrogens at least in the circulation, to influence bone development and/or

maintenance. It thus seems likely that further elucidation of the regulation steroid metabolism and the potential mechanisms by which androgenic and estrogenic effects are coordinated, will have physiological, pathophysiological and therapeutic implications.

MECHANISMS OF ANDROGEN ACTION IN BONE: THE ANDROGEN RECEPTOR

The specific mechanisms by which androgens affect skeletal homeostasis, and whether these effects are directly mediated in bone, are areas of intensified research. As a classic steroid hormone, the biological cellular signaling responses to androgen are mediated through the AR, a ligand-inducible transcription factor. ARs have been identified in a variety of cells found in bone [55] and the characterization of AR expression in these cells thus clearly identifies bone as a target tissue for androgen action. The direct effects of androgen that influence the complex processes of proliferation, differentiation, mineralization and gene expression in the osteoblast are being characterized, but much remains to be established. Androgen effects on bone may also be indirectly modulated and/or mediated by other autocrine and paracrine factors in the bone microenvironment. The rest of this chapter will review recent progress on the characterization of androgen action in bone.

MOLECULAR MECHANISMS OF ANDROGEN ACTION IN BONE CELLS: THE AR

Direct characterization of AR expression in a variety of tissues, including bone, was made possible by the cloning of the AR cDNA. The AR is a member of the class I (so-called classical or steroid) nuclear receptor superfamily, as are the ERα and ERβ isoforms, the progesterone receptor, the mineralocorticoid and glucocorticoid receptor. Steroid receptors are transcription factors with a highly-conserved modular design characterized by three functional domains: the transactivation, DNA binding and ligand binding domains. In the absence of ligand, the AR protein is generally localized in the cytoplasmic compartment of target cells in a large complex of molecular chaperones, consisting of loosely bound heat-shock, cyclophilin and other accessory proteins. As lipids, androgens can freely diffuse through the plasma membrane to bind the AR and induce a conformational change. Once bound by ligand, the AR dissociates from the multiprotein complex, translocates to the nucleus and recruits co-activators or co-repressors, some of which are cell type specific [56], allowing the formation

of homodimers (or potentially heterodimers) that activate a cascade of events in the nucleus. Once bound to DNA, the AR influences transcription and/or translation of a specific network of genes, leading to cell-specific responses to the steroid.

p0190   A steroid hormone target tissue can be defined as one that possesses the steroid receptor, at a functional level, with a measurable response in the presence of hormone. In addition to other organ systems including muscle, brain, liver, kidney, fat and prostate, bone tissue clearly meets this standard with respect to androgen. Colvard et al first reported the presence of AR mRNA and specific androgen binding sites in normal human osteoblastic cells [57]. The abundance of both AR and ER proteins was similar, suggesting that androgens and estrogens each play important roles in skeletal physiology. Subsequent reports have confirmed AR mRNA expression and/or the presence of androgen binding sites in both normal and clonal, transformed osteoblastic cells derived from a variety of species. The size of the AR mRNA transcript in osteoblasts (about 10 kb) is similar to that described in prostate and other tissues, as is the size of the AR protein analyzed by Western blotting (≈ 110 kDa) [18]. There are reports of two isoforms of AR protein in human osteoblast-like cells (≈ 110 and ≈ 97 kDa) [58] as first described in human prostatic tissue. It appears these isoforms do not possess similar functional activities in bone, particularly with respect to effects on proliferation [59]. The number of specific androgen binding sites in osteoblasts varies, depending on methodology and the cell source, from 1000 to 14000 sites/cell, but is in a range seen in other androgen target tissues. Furthermore, the binding affinity of the AR found in osteoblastic cells ($K_d = 0.5$–2×10^{-9}) is typical of that found in other tissues. Androgen binding is specific, without significant competition by estrogen, progesterone or dexamethasone [18, 57, 60]. Finally, testosterone and DHT appear to have relatively similar binding affinities [18, 61]. All these data are consistent with the notion that the direct biologic effects of androgenic steroids in osteoblasts are mediated at least in part via classic mechanisms associated with the AR as a member of the steroid hormone receptor superfamily described above.

p0200   In addition to the classical AR present in bone cells, several other androgen-dependent signaling pathways have been reported. Specific binding sites for weaker adrenal androgens (such as DHEA) have been described [62]; DHEA does transactivate AR [11], thus raising the possibility that DHEA or similar androgenic compounds may also have direct effects in bone. DHEA and its metabolites may also bind and activate additional receptors, including ER, peroxisome proliferator activated receptor-α and pregnane X receptor. Bodine et al [63] showed that DHEA caused a rapid inhibition of c-*fos* expression in human osteoblastic cells that was more robust than seen with the classical androgens (DHT, testosterone, androstenedione).

In addition, DHEA may inhibit bone resorption by osteoclasts when in the presence of osteoblasts, likely through changes in osteoprotgerin (OPG) and receptor activator of NFκB ligand (RANKL) concentrations [64]. Alternatively, androgens may be specifically bound in osteoblastic cells by a novel 63-kDa cytosolic protein [65]. In addition, there are reports of distinct AR polymorphisms identified in different races that may have biological impact on androgen responses [66]. These different isoforms have the potential to interact in distinct fashions with other signaling molecules, such as c-Jun [67], but to date none has been shown to affect bone tissue. Finally, androgens may regulate osteoblast activity via rapid non-genomic mechanisms [68] through membrane receptors displayed at the bone cell surface [69]. The role and biologic significance of these non-classical signaling pathways in androgen-mediated responses in bone in vivo remains highly controversial and most data support the contention that genomic signaling is the more significant regulator in bone and other tissues [70–74].

LOCALIZATION OF AR EXPRESSION IN OSTEOBLASTIC POPULATIONS   s0100

p0210 Ultimately, bone mass is determined by two biological processes: formation and resorption. Distinct cell types mediate these processes. The bone-forming cell, the osteoblast, synthesizes bone matrix, regulates mineralization and is responsive to most calciotropic hormones. The osteoclast is responsible for bone resorption. Clues about the potential sequelae of AR signaling in bone will likely be derived from a better understanding of the cell types in which expression is documented. In vivo analysis has demonstrated significant expression of AR in all cells of the osteoblast lineage including osteoblasts, osteocytes and osteoclasts [75]. Interestingly, ARs are also expressed in bone marrow stromal [76] and mesenchymal precursor cells [77], pluripotent cells that can differentiate into muscle, bone and fat. Androgen action may modulate precursor differentiation toward the osteoblast and/or myoblast lineage, while inhibiting differentiation toward the adipocyte lineage. These effects on stromal differentiation could underlie some of the well-described consequences of androgen administration on body composition including increased muscle mass. To date, it has not been established how significant the contribution is of the increased muscle mass associated with androgen administration positively to influence bone quality. However, anabolic steroid therapy to severely burned children results in significantly increased lean mass months before an effect can be demonstrated in bone [78], suggesting the importance of muscle in biomechanical linkage as a mechanism to increase bone mineral density.

p0220 In the bone microenvironment, the localization of AR expression has been described in intact human bone by Abu et al using immunocytochemical methods [55]. In developing bone from young adults, ARs were predominantly expressed in active osteoblasts at sites of bone formation (Figure 25.2). ARs were also observed in osteocytes embedded in the bone matrix. Importantly, both the pattern of AR distribution and the level of expression were similar in males and in females. Furthermore, AR is observed in bone marrow and stromal/osteoblast precursor cells [76]. In addition, expression of the AR has been characterized in cultured osteoblastic cell populations isolated from bone biopsy specimens, determined at both the mRNA level and by binding analysis [58]. Expression varied according to the skeletal site of origin and age of the donor of the cultured osteoblastic cells: AR expression was higher at cortical and intramembranous bone sites and lower in trabecular bone. This distribution pattern correlates with androgen responsiveness in the bone compartment. AR expression was highest in osteoblastic cultures generated from young adults and somewhat lower in samples from either prepubertal or senescent bone. Data indicate preferential nuclear staining
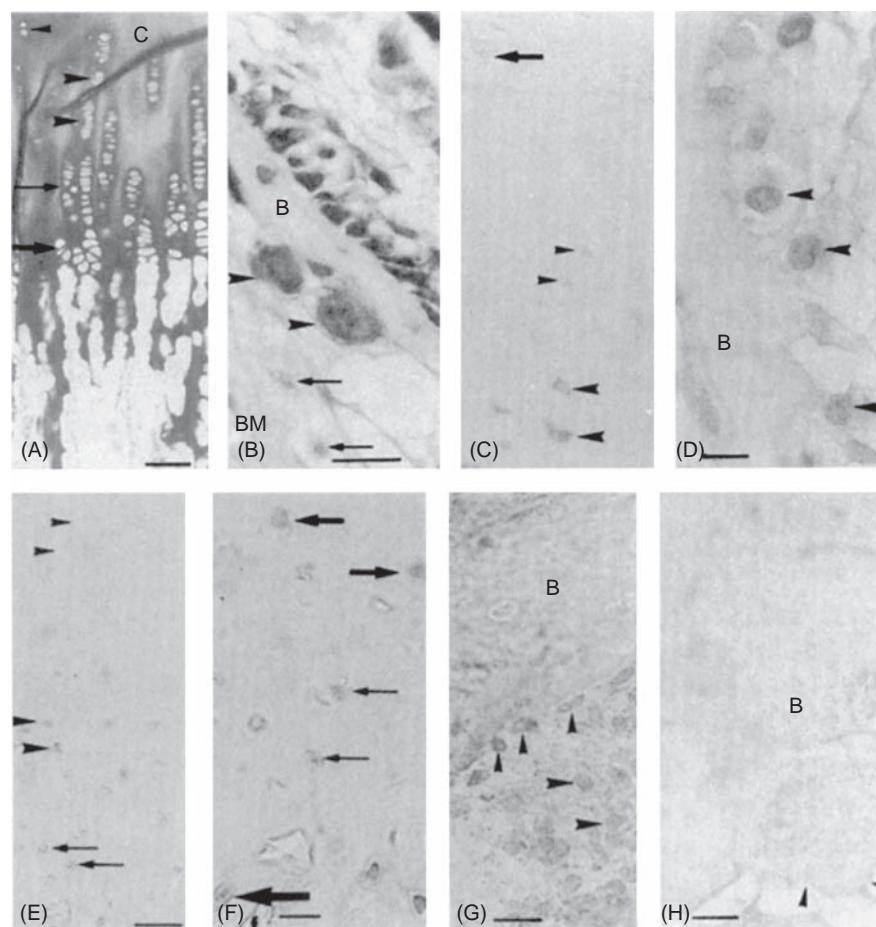
f0020 **FIGURE 25.2** The localization of AR in normal tibial growth plate and adult osteophytic human bone. (A) Morphologically, sections of the growth plate consist of areas of endochondral ossification with undifferentiated (small arrow head), proliferating (large arrow heads), mature (small arrow) and hypertrophic (large arrow) chondrocytes. Bar = 80μm. An inset of an area of the primary spongiosa is shown in (B). (B) Numerous osteoblasts (small arrow heads) and multinucleated osteoclasts (large arrow heads) on the bone surface. Mononuclear cells within the bone marrow are also present (arrows). Bar = 60μm. (C) In the growth plate, AR is predominantly expressed by hypertrophic chondrocytes (large arrow heads). Minimal expression is observed in the mature chondrocytes (small arrow heads). The receptors are rarely observed in the proliferating chondrocytes (arrow). (D) In the primary spongiosa, the AR is predominantly and highly expressed by osteoblasts at modeling sites (arrow heads). Bar = 20μm. (E) In the osteophytes, AR is also observed at sites of endochondral ossification in undifferentiated (small arrow heads), proliferating (large arrow heads), mature (small arrows) and hypertrophic-like (large arrow) chondrocytes. Bar = 80 μm. (F) A higher magnification of (E) showing proliferating, mature and hypertrophic-like chondrocytes (large arrows, small arrows, and very large arrows respectively) Bar = 40μm. (G) At sites of bone remodeling, the receptors are highly expressed in the osteoblasts (small arrow heads) and also in mononuclear cells in the bone marrow (large arrow heads). Bar = 40μm. (H) AR is not detected in osteoclasts (small arrow heads) Bar = 40μm. B, Bone: C, Cartilage; BM, Bone marrow [55]

of AR in males at sexual maturity, suggesting activation and translocation of the receptor in bone when androgenic steroid levels are elevated, consistent with androgen regulation of AR levels [79,80]. Again, no differences were found between male and female samples, suggesting that differences in receptor number per se do not underlie development of a sexually dimorphic skeleton. Since androgens are so important in bone development at the time of puberty, it is not surprising that ARs are also present in epiphyseal chondrocytes [55]. The expression of ARs in such a wide variety of cell types known to be important for bone modeling during development and remodeling in the adult, provides evidence for direct actions of androgens in bone and cartilage tissue. These results illuminate the complexity of androgen action on bone. Thus, although bone is a target tissue with respect to androgen action, the mechanisms and cell types by which androgens exert their effects on bone biology remain incompletely characterized. In terms of mechanism of action, an additional complexity is that testosterone may influence bone directly by activation of the AR

or indirectly after aromatization into estrogens with subsequent activation of ER.

REGULATION OF AR EXPRESSION

The regulation of AR expression in osteoblasts is also incompletely characterized. Autologous regulation of AR mRNA by androgen has been well described and appears to be tissue specific; upregulation by androgen exposure is seen in a variety of mesenchymal cells including osteoblasts [79–81], whereas in prostate and smooth muscle tissue, downregulation is observed after androgen exposure [79] (Figure 25.3). The androgen mediated upregulation observed in osteoblasts can occur through changes in AR gene transcription [79,80]. Interestingly, a novel property of the AR is that binding of androgen can increase AR protein levels, as shown in osteoblastic cells [80]. This property distinguishes AR from most other steroid receptor molecules that are
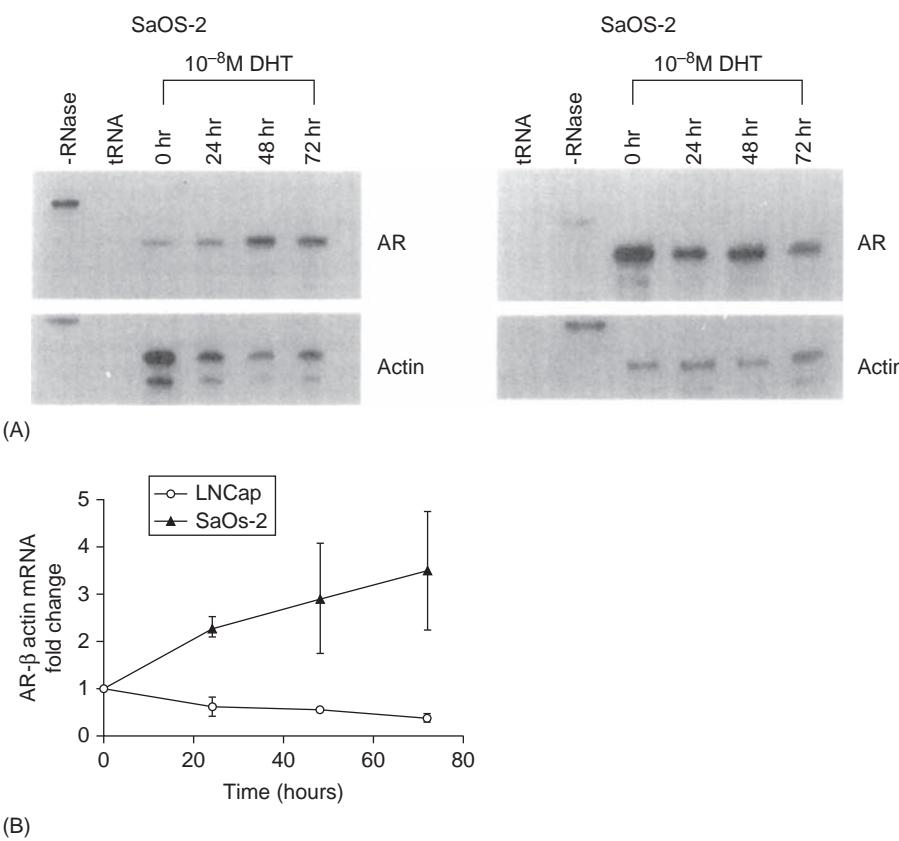
FIGURE 25.3   Dichotomous regulation of AR mRNA levels in osteoblast-like and prostatic carcinoma cell lines after exposure to androgen. (A) Time course of changes in AR mRNA abundance after DHT exposure in human SaOS-2 osteoblastic cells and human LNCaP prostatic carcinoma cells. To determine the effect of androgen exposure on hAR mRNA abundance, confluent cultures of either osteoblast-like cells (SaOS-S) or prostatic carcinoma cells (LNCaP) were treated with 10^{-8} M DHT for 0, 24, 48, or 72h. Total RNA was then isolated and subjected to RNase protection analysis with $50\mu g$ total cellular RNA from SaOS-2 osteoblastic cells and $10\mu g$ total RNA from LNCaP cultures. (B) Densitometic analysis of AR mRNA steady-state levels. The AR mRNA to β-actin ratio is expressed as the mean ± SEM compared to the control value from three to five independent assessments [79]

downregulated by ligand binding. At least in part, the elevated AR protein levels may be a consequence of increased stability mediated by androgen binding [82], but the stability of AR in osteoblastic cells has not been determined. The mechanism(s) that underlie tissue specificity in autologous AR regulation and the possible biological significance of distinct autologous regulation of AR, is not yet understood. It is possible that AR upregulation by androgen in bone may result in an enhancement of androgen responsiveness at times when androgen levels are rising or elevated. Ligand-independent activation of AR has also been described in other tissues, but has not been explored in bone.

Quantitative determination of AR expression during osteoblast differentiation is difficult to achieve in bone slices. However, analysis of AR, ERα and ERβ mRNA and protein expression during osteoblast differentiation in vitro demonstrates that each receptor displays distinct differentiation stage patterns in osteoblasts (Figure 25.4) [83], indicating that osteoblast differentiation and steroid receptor regulation are intimately associated. The levels of AR expression increase throughout osteoblast differentiation with the highest AR levels seen in mature osteoblast or osteocyte cultures. Given the high level of AR expression, this finding suggests that an important compartment for androgen action in bone may be mature, mineralizing osteoblasts. Given that the osteocyte is the most abundant cell type in bone and a likely mediator of both focal bone deposition and the response to mechanical strain [84], it is not surprising that androgens may also augment the osteoanabolic effects of mechanical strain in osteoblasts [85]. However, an analysis of the consequences of AR action on loading in vivo has yet to be performed.

AR expression in osteoblasts can be upregulated by exposure to other steroid hormones, including glucocorticoids, estrogen or 1,25-dihydroxyvitamin D_3 [60]. Whether additional hormones, growth factors or agents influence AR expression in bone is not known. Further, whether the AR in osteoblasts undergoes post-translational processing that might influence receptor signaling (stabilization, phosphorylation, etc.) as described in other tissues [86,87] and the potential functional implications [88] are also unknown.

Steroid receptor transcriptional activity, including that of the AR, is strongly influenced by transcriptional regulators such as co-activators or co-repressors. These co-activators/co-repressors can influence the downstream signaling of nuclear receptors; their levels are influenced by the cellular context and these co-reguators can differentially affect specific promoters. AR specific co-activators have been identified [89], many of which interact with the ligand binding domain of the receptor. Expression and regulation of these modulators may thus influence the ability of steroid receptors to regulate gene expression in bone, but this remains underexplored with respect to androgen receptor action. Furthermore, phosphorylation of specific co-activators, for

(A)

(B)
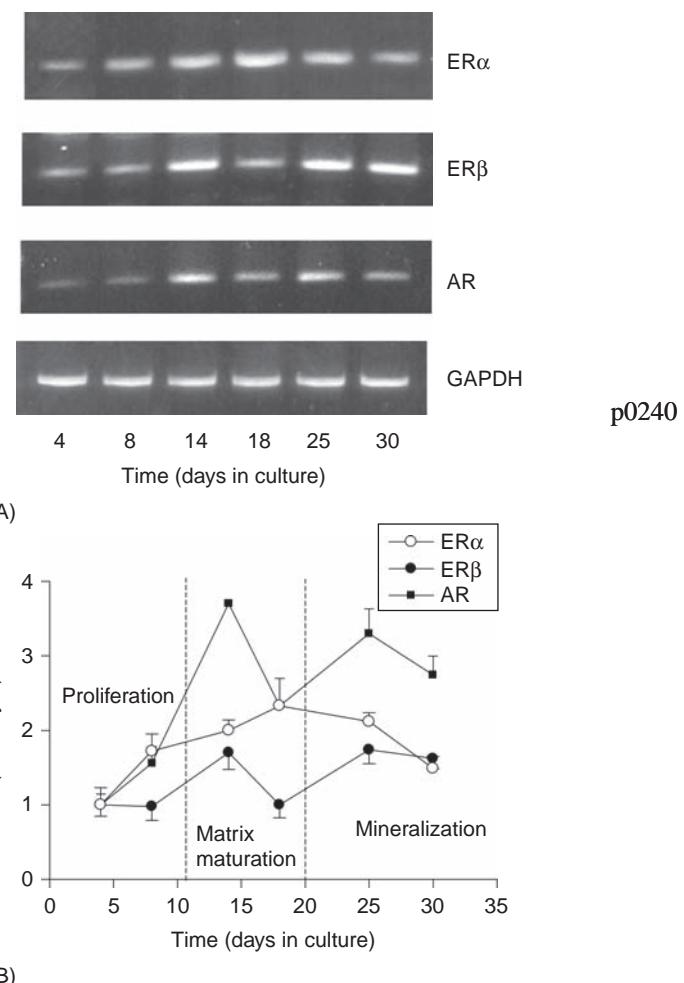
FIGURE 25.4 Expression analyses of ERα, ERβ and AR during in vitro differentiation in normal rat osteoblastic (rOB) cultures. (A) Normal rOB cells were cultured for the indicated number of days during proliferation, matrix maturation, mineralization and post-mineralization stages. Total RNA was isolated and subjected to relative RT-PCR analysis using primers specific for rat ERα, ERβ and AR or rat GAPDH. Reverse transcription was conducted with PCR carried out for 40 cycles for the steroid receptors, with parallel reactions performed using GAPDH primers for 25 cycles (all in the linear range). Bands for rat ERα at the predicted 240 bp, rat ERβ at 262 bp, rat AR at 276 bp and GAPDH at 609 bp are shown. (B) Analyses of ERα, ERβ and AR mRNA relative abundance. Semiquantitative analysis of mRNA steady-state expression by relative RT-PCR was performed after scanning the negative image of the photographed gels. Data are expressed in arbitrary units as the ratio of receptor abundance to GAPDH expression, then normalized to expression values at day 4 in pre-confluent cultures. Data represent mean $\pm$ SEM [83]

example by mitogen-activated (MAP) kinase, may influence AR activity at specific target genes [90]. The specific co-activator/co-repressor profile present in cells representing different bone compartments (i.e. periosteal fibroblasts, proliferating or mineralizing osteoblasts) may help

determine the activity of the selective receptor modulators such as SARMS described above.

EFFECTS OF ANDROGENS ON THE PROLIFERATION AND DIFFERENTIATION OF OSTEOBLASTIC CELLS

p0270   Evidence suggests that androgens act directly on the osteoblast to influence expression and function. However, and likely as a consequence of the complexity of osteoblast differentiation, data supporting an effect of androgen to influence bone cells are inconsistent. There are reports, some in clonal osteoblastic cell lines, of modulatory effects of gonadal androgen treatment on proliferation, differentiation, matrix production and on mineral accumulation [91], but the effect of androgen on osteoblast proliferation has been shown to be biphasic in nature, with enhancement following short or transient treatment but significant inhibition following longer treatment. As a case in point, Kasperk et al [92] demonstrated in osteoblast-like cells in primary culture (murine, passaged human) that a variety of androgens in serum-free medium increase DNA synthesis ([^{3}H]thymidine incorporation) and cell counts. Testosterone and non-aromatizable androgens (DHT and fluoxymesterone) were nearly equally effective regulators. Yet the same group [60] reported that prolonged DHT treatment inhibited normal human osteoblastic cell proliferation (cell counts) in cultures pretreated with DHT. In addition, Benz et al have shown that prolonged androgen exposure in the presence of serum inhibited proliferation (cell counts) by 15–25% in a transformed human osteoblastic line (TE-85) [61]. Testosterone and DHT again were nearly equally effective regulators. Hofbauer et al [93] examined the effect of DHT exposure on proliferation in hFOB/AR-6, an immortalized human osteoblastic cell line stably transfected with an AR expression construct (with ≈4000 receptors/cell). In this line, DHT treatment inhibited cell proliferation by 20–35%. Although various studies employed different model systems (transformed osteoblastic cells versus second to fourth passage normal human cells) and culture conditions (including differences in the state of osteoblast differentiation, receptor number, phenol red-containing versus phenol red-free or serum containing versus serum-free), it appears exposure time may be the significant variable. Time dependence for the response to androgen was clearly shown by Wiren et al [94], where osteoblast proliferation was stimulated with brief treatment times, but with prolonged DHT treatment that is more consistent with an in vivo exposure, osteoblast viability decreased (Figure 25.5). This result was AR dependent (i.e. inhibitable by co-incubation with flutamide) and was observed in both normal rat calvarial osteoblasts and in AR stably transfected MC-3T3 cells. In mechanistic terms, reduced viability was associated with overall
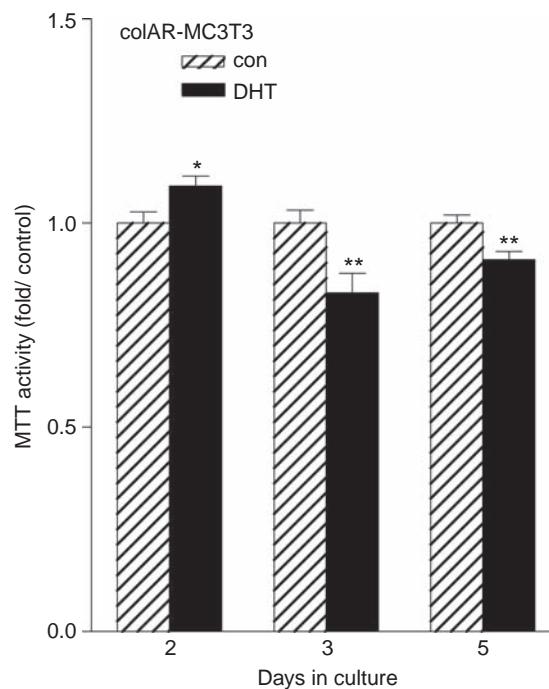
FIGURE 25.5   Complex effect of androgen on DNA accumulation in osteoblastic cultures. Kinetics of DHT response in proliferating colAR-MC3T3 cultures measured with colorimetric (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay. Cultures of stably transfected colAR-MC3T3 continuously with 10^{-8} M DHT for 2 days led to increased MTT accumulation, but longer treatment for 3 or 5 days resulted in inhibition. Data are mean $\pm$ SEM of six to eight dishes with six wells/dish. *$P < 0.05$; **$P < 0.01$ (versus control) [94]

f0050

reduction in MAP kinase signaling and with downstream inhibition of *elk-1* gene expression, protein abundance and extent of phosphorylation. The inhibition of MAP kinase activity after chronic androgen treatment again contrasts with the response seen after brief androgen exposure, where stimulation of MAP kinase signaling and AP-1 transactivation is observed [94]. This rapid in vitro response may be mediated through non-genomic mechanisms [95,96]. Combined, most data suggest that the in vivo response to androgen treatment on osteoblast proliferation is generally inhibitory.

p0280   It is also important to consider the process of programmed cell death, or apoptosis, as a component of control of osteoblast survival. In particular, as the osteoblast population differentiates in vitro, the mature bone cell phenotype undergoes apoptosis [97]. With respect to the effects of androgen exposure, chronic DHT treatment has been shown to result in enhanced osteoblast apoptosis in vitro in both proliferating osteoblastic (at day 5) and in mature osteocytic cultures (day 29) (Figure 25.6) [98]. In the same study, the inhibition observed with DHT treatment was opposite to inhibitory effects on apoptosis seen with E$_2$ treatment. An androgen-mediated increase in the Bax/Bcl-2 ratio was also observed, predominantly through
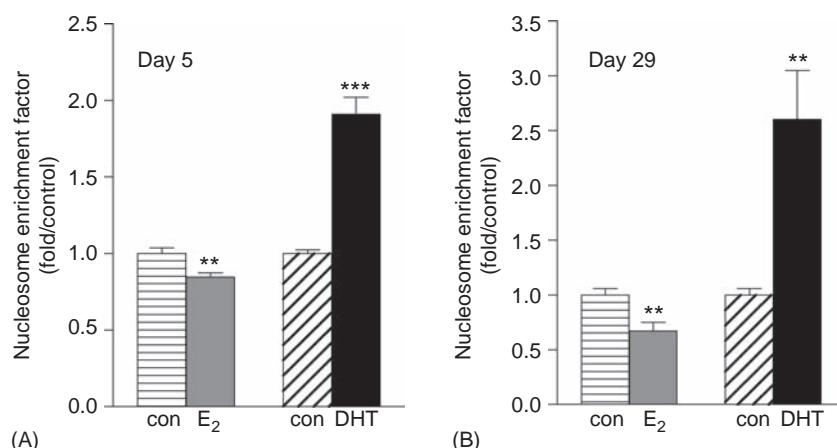
FIGURE 25.6 Characterization of osteoblast apoptosis: results of androgen and estrogen treatment during proliferation (day 5) and during differentiation into mature osteoblast/osteocytes cultures (day 29). Apoptosis was assessed at day 5 or day 29 after continuous DHT and E_2 treatment (both at 10^{-8} M). Apoptosis was induced by etoposide treatment in proliferating cultures and by serum starvation for 48 h in confluent cultures before isolation, replaced with 0.1% BSA. (A) Analysis of apoptosis after evaluating DNA fragmentation by cytoplasmic nucleosome enrichment at day 5. The data are expressed as mean $\pm$ SEM (n = 6) from two independent experiments. **$P < 0.01$, ***$P < 0.001$ (versus control). (B) Analysis of apoptosis by cytoplasmic nucleosome enrichment analysis at day 29. The data are expressed as mean $\pm$ SEM (n = 6) from two independent experiments. **$P < 0.01$ versus control [98]

inhibition of Bcl-2 and was dependent on functional AR. Overexpression of *bcl-2* or RNAi knockdown of *bax* abrogated the effects of DHT, indicating that increased Bax/Bcl-2 was necessary and sufficient for androgen-enhanced apoptosis. The increase in the Bax/Bcl-2 ratio was at least in part a consequence of reductions in Bcl-2 phosphorylation and protein stability, consistent with inhibition of MAP kinase pathway activation after DHT treatment as noted above. Supporting in vivo analysis of calvaria in AR-transgenic male mice also demonstrated enhanced apoptosis with elevated TUNEL staining in both osteoblasts and osteocytes and was observed even in areas of new bone growth [98]. This may not be surprising, given an association between new bone growth and apoptosis [99], as has been observed in other remodeling tissues and/or associated with development and tissue homeostasis. Apoptotic cell death could thus be important in making room for new bone formation and matrix deposition, which may have clinical significance by influencing bone homeostasis and bone mineral density [100]. Thus, mounting evidence suggests that chronic androgen treatment does not increase osteoblast number or viability in the mature bone compartment and this evidence does not support a suggestion for strong anabolic responses directly in bone as a consequence of androgen therapy.

p0290   Osteoblast differentiation can be characterized by changes in alkaline phosphatase activity and/or alterations in the expression of important extracellular matrix proteins, such as type I collagen, osteocalcin and osteonectin. As noted with other responses, the effects of androgens on expression of these marker activities/proteins are poorly described and inconsistent. For example, enhanced osteoblast differentiation, as measured by increased matrix production, has been shown to result from androgen exposure in both normal osteoblasts and transformed clonal human osteoblastic cells (TE-89). Androgen treatment appeared to increase the proportion of cells expressing alkaline phosphatase activity, thus representing a shift toward a more differentiated phenotype [101]. Kasperk et al subsequently reported dose-dependent increases in alkaline phosphatase activity in both high and low-alkaline phosphatase subclones of SaOS2 cells [102] and human osteoblastic cells [60]. However, there are also reports, in a variety of model systems, of androgens either inhibiting [93] or having no effect on alkaline phosphatase activity [81], which may reflect both the complexity and dynamics of osteoblastic differentiation. Androgen-mediated increases in type I α-1 collagen protein and mRNA levels [61, 102] and increased osteocalcin secretion [60], have also been described. Consistent with increased collagen production, androgen treatment has also been shown to stimulate mineral accumulation in a time- and dose-dependent manner [60, 81, 103]. However, in vivo models with bone-targeted overexpression of AR in transgenic mice, employing two distinct promoters, showed decreased levels of bone markers in total RNA extracts derived from long bone samples [27, 104]. Both lines demonstrate decreased levels of most osteoblastic and most osteoclastic genes that include reduced levels of the major matrix proteins collagen, osterix and osteocalcin. Combined, these results suggest that, under certain conditions, androgens may enhance osteoblast differentiation. However, it is becoming increasingly clear that the direct effects of androgens on osteoblasts are generally negative and thus likely play an inhibitory role in the regulation of bone matrix production and/or organization.

Positive anabolic effects of androgen in bone may thus be limited to distinct lineages, for example cells in the periosteal compartment [27,104].

ANDROGEN EFFECTS ON OSTEOCLASTS AND OTHER CELLS

Androgens also influence other cell types in bone that are important in determining bone balance. Potential modulation of osteoclast action by DHT is incompletely characterized, although there are reports of AR expression in the osteoclast [75]. Androgen treatment reduces bone resorption of isolated osteoclasts, inhibits osteoclast formation [105] and that stimulated by parathyroid hormone (PTH) [106] and may play a direct role regulating aspects of osteoclast activity in both AR null mice [107]. Indirect effects of androgen to modulate osteoclasts are indicated by the increase in OPG following testosterone treatment in osteoblasts [108] and in skeletally-targeted AR-transgenic mice [27]. In addition, DHEA treatment has been shown to decrease in the OPG/RANKL ratio in osteoblastic cells and inhibit osteoclast activity in coculture [64]. Androgen may be a less significant determinant of bone resorption in vivo than estrogen [109], although this remains controversial [110].

As with effects noted in osteoblastic populations, androgens regulate chondrocyte proliferation and expression. Although some of the consequences of androgen action are mediated after metabolic conversion to estrogen, which limits long bone growth, non-aromatizable androgen stimulates longitudinal bone growth [111]. AR expression has been demonstrated in cartilage and androgen exposure promotes chondrogenesis. Increased [^{35}S]sulfate incorporation into newly synthesized cartilage [112] is androgen mediated. Regulation of these effects is obviously complex, as they were dependent on the age of the animals and the site from which chondrocytes were derived. Thus, in addition to effects on osteoblasts, multiple cell types in the skeletal milieu are regulated by androgen exposure.

The effects of androgens on osteoblast activity must certainly also be considered in the context of the very complex endocrine, paracrine and autocrine milieu in the bone microenvironment. Systemic and/or local factors can act in concert, or can antagonize, to influence bone cell function. This has been well described with regard to modulation of the effects of estrogen on bone. Androgens have also been shown to regulate well-known modulators of osteoblast proliferation or function. The most extensively characterized growth factor influenced by androgen exposure is transforming growth factor-β (TGF-β). TGF-β is stored in bone (the largest reservoir for TGF-β) in a latent form and has been show to be a mitogen for osteoblasts [113]. Androgen treatment has been shown to increase TGF-β activity in human osteoblast primary cultures. The expression of some TGF-β mRNA transcripts (apparently TGF-β2) was increased, but no effect on TGF-β1 mRNA abundance was observed [63,92]. At the protein level, specific immunoprecipitation analysis reveals DHT mediated increases in TGF-β activity to be predominantly TGF-β2 [60,63]. DHT has also been shown to inhibit both TGF-β gene expression and TGF-β-induced early gene expression that correlates with growth inhibition in this cell line [93]. The TGF-β-induced early gene has been shown to be a transcription factor that may mediate some TGF-β effects. These results are consistent with the notion that TGF-β may mediate androgen effects on osteoblast proliferation. On the other hand, TGF-β1 mRNA levels are increased by androgen treatment in human clonal osteoblastic cells (TE-89), under conditions where osteoblast proliferation is slowed [61]. Thus, the specific TGF-β isoform may determine osteoblast responses. It is interesting to note that, in vivo, orchiectomy (ORX) drastically reduces bone content of TGF-β levels and testosterone replacement prevents this reduction [114]. These data support the findings that androgens influence cellular expression of TGF-β and suggest that the bone loss associated with castration is related to a reduction in growth factor abundance induced by androgen deficiency.

Other growth factor systems may also be influenced by androgens. Conditioned media from DHT treated normal osteoblast cultures are mitogenic and DHT pretreatment increases the mitogenic response to fibroblast growth factor and to insulin- like growth factor II (IGF-II) [92]. In part, this may be due to slight increases in IGF-II binding in DHT treated cells [92], as IGF-I and IGF-II levels in osteoblast conditioned media are not affected by androgen [92]. Although most studies have not found regulation of IGF-I or IGF-II abundance by androgen exposure [18,92], there is a report in an androgen-responsive human osteoblastic cell line that androgens can increase IGF-I, IGF binding protein (IGFBP)-2 and IGFBP-3 expression and, at the same time, decrease levels of the inhibitory IGFBP-4 [115]. Androgens may also modulate expression of components of the AP-1 transcription factor [63] and/or transiently increase AP-1 transcriptional activation [94]. Thus, androgens may modulate osteoblast differentiation via a mechanism whereby growth factors or other mediators of differentiation are regulated by androgen exposure.

Androgens may also modulate responses to additional important osteotropic hormones/regulators. Testosterone and DHT specifically inhibit the cAMP response elicited by PTH or parathyroid hormone-related protein (PTHrP) in the human clonal osteoblast-like cell line SaOS-2 while the inactive or weakly active androgen 17α-epitestosterone had no effect. This inhibition may be mediated via an effect on the PTH receptor-G$_s$-adenylyl cyclase [116]. The production of prostaglandin E$_2$ (PGE$_2$), another important regulator of bone metabolism, is also affected by androgens. Pilbeam and Raisz showed that androgens (both DHT and testosterone) were potent inhibitors of both parathyroid

hormone and interleukin-1 stimulated PGE₂ production in cultured neonatal mouse calvaria [117]. The effects of androgens on PTH action and PGE₂ production suggest that androgens could act to modulate (reduce) bone turnover in response to long-term treatment with these agents.

p0350   Finally, both estrogen and androgen [118] can inhibit production of interleukin-6 by osteoblastic cells. In stromal cells of the bone marrow, androgens have been shown to have potent inhibitory effects on the production of interleukin-6 and the subsequent stimulation of osteoclastogenesis by marrow osteoclast precursors [119]. Interestingly, adrenal androgens (androstenediol, androstenedione, DHEA) have similar inhibitory activities on interleukin-6 gene expression and protein production by stromal cells [119]. The loss of inhibition of interleukin-6 (IL-6) production by androgen may also contribute to the marked increase in bone remodeling and resorption that follows orchiectomy, in addition to modulation of osteoclast activity through changes in the OPG/RANKL ratio as noted above. Moreover, androgens inhibit the expression of the genes encoding the two subunits of the IL-6 receptor (gp80 and gp130) in the murine bone marrow, another mechanism which may blunt the effects of this osteoclastogenic cytokine in intact animals [120]. In these aspects, the effects of androgens seem to be very similar to those of estrogen, which may also inhibit osteoclastogenesis via mechanisms that involve IL-6 inhibition and/or
AUQ1   OPG/RANKL ratio changes.

s0140   ANDROGEN EFFECTS ON BONE: ANIMAL STUDIES

p0360   The effects of androgens on bone remodeling have been examined fairly extensively in animal models. Much of this work has been in species not perfectly suited to reflect human bone metabolism (rodents) and, certainly, the field remains incompletely explored. Nevertheless, animal models do provide valuable insights into the effects of androgens at organ and cellular levels. Many of the studies of androgen action have been performed in male rodents, in which rapid skeletal growth occurs until about 4 months of age, at which time epiphyseal growth slows markedly (although never completely ceases at some sites). Because the effects of androgen may be different in growing and more mature animals [121], it is appropriate to consider the two situations independently.

EFFECTS ON BONE GROWTH DURING SKELETAL DEVELOPMENT

In most mammals, there is a marked gender difference in bone morphology. The mechanisms responsible for these differences are complex and presumably involve both androgenic and estrogenic actions. During early development and adolescence, skeletal development is characterized by marked expansion of cortical proportions and increasing trabecular density. During this process, the skeleton develops distinctly in males and females, with the most significant differences at the periosteal and endocortical surfaces. As sex differences in skeletal morphology and physiology occur at or around puberty, it is hypothesized that gender differences, particularly with respect to 'bone quality' and architecture, i.e. predominantly bone width, are modulated at least in part by estrogens and androgens. Consistent with this, a distinct response to estrogen and androgen has been described in vivo especially in cortical bone. At the periosteum, estrogen suppresses while androgen stimulates new bone formation yet, conversely, at the endocortical surface, estrogen stimulates but androgen strongly suppresses formation, at least in males [27, 104]. Thus, estrogen tends to decrease while androgen increases radial growth in cortical bone through periosteal apposition. At the endocortical surface, in contrast, estrogen increases while androgen suppresses bone formation [27, 104]. These distinct responses to estrogen and androgen during growth likely play an important role in determining sexual dimorphism of the skeleton, i.e. that male bones are wider but not thicker than females [122]. Young men do have larger bone areas than women with increased whole bone cross-sectional area, particularly at peripheral sites [123]. Interestingly aromatase gene polymorphisms that variably influence sex steroid concentrations also suggest a positive role for androgen action at the periosteal surface, given findings that testosterone concentrations were associated with larger bones and larger medullary space while, in contrast, estradiol levels were shown to be associated with smaller medullary space [124]. Androgens are also essential for the production of peak total-body bone mass in males [125]. Low levels of estrogen (with increased levels of androgen) may also be important for stimulation of periosteal bone formation during development [40]. Yet, in sum, these two sex steroids may act in opposition at distinct bone sites/compartments.

EFFECTS ON EPIPHYSEAL FUNCTION DURING DEVELOPMENT   s0160

p0380   Estrogens are particularly important for the regulation of epiphyseal function and act to reduce the rate of longitudinal growth via influences on chondrocyte proliferation and action, as well as on the timing of epiphyseal closure
s0150   [126]. Androgens appear to have opposite effects and tend to promote long bone growth, chondrocyte maturation and metaphyseal ossification. Androgen deficiency retards
p0370   those processes [127]. Nevertheless, excess concentrations

of androgen will accelerate aging of the growth plate and reduces growth potential, possibly via conversion to estrogens.

p0390 Although the specific roles of sex steroids in the regulation of epiphyseal growth and maturation remain somewhat unresolved, there is evidence that androgens do have direct effects independent of those of estrogen. For instance, testosterone injected directly into the growth plates of rats increases plate width [128]. In a model of endochondral bone development based on the subcutaneous implantation of demineralized bone matrix in castrate rats, both testosterone and DHT increase the incorporation of calcium during osteoid formation [103]. Interestingly, in this model, androgens reduced the incorporation of [^{35}S]sulfate into glycosaminoglycans early in the developing cartilage. In sum, these data support the contention that androgens play a direct role in chondrocyte physiology.

s0170 **EFFECTS ON BONE MASS IN GROWING MALE ANIMALS: ANIMAL MODELS OF ALTERED ANDROGEN RESPONSIVENESS**

p0400 The most dramatic effect of androgens during growth is on bone size. Male animals have larger bones and, particularly, thicker cortices than females [126]. The contribution of AR signaling in vivo has been approached in genetic animal models with global AR modulation, including global (i.e. non-targeted) AR knockout mice [129] and the testicular feminization (Tfm) model of androgen insensitivity syndrome (AIS) [130,131]. In all of these models, the effect of the genetic manipulation is present from before birth. The bone phenotype that develops in a global AR null (ARKO) male mouse model is a high-turnover osteopenia, with reduced trabecular bone volume and a significant stimulatory effect on osteoclast function with little effect in females [129,132,133]. In the Tfm (AR deficient), androgens are presumed to be incapable of action, but estrogen and androstenedione concentrations are considerably higher than in normal males [134,135]. Clear increases also exist in Tfm male rats in serum concentrations of calcium, phosphorus and osteocalcin, whereas IGF-I concentrations are decreased. Estimates of bone mass suggest that Tfm rats have reduced longitudinal and radial growth rates, but that trabecular volume and density are similar to those of normal rats. In selected sites, measures of bone mass and remodeling were intermediate between normal male and female values. This model indicates that androgens have an independent role to play in normal bone growth and metabolism, but the model is complex and not easily dissected. In Tfm mice, meticulous analysis by Vanderschueren et al [130] has shown that the positive effects of testosterone on cortical bone are generally mediated by stimulation of periosteal p0420 bone formation, which was absent in Tfm mice. The analysis

shows that AR-mediated testosterone action is essential for periosteal bone formation (in male mice) and also contributes to trabecular bone maintenance. This is very similar to the study of humans with the androgen insensitivity syndrome. Marcus et al [54] reported that there is a deficit in bone mineral density in women with androgen insensitivity even when compliance with estrogen replacement is excellent. However, inadequate estrogen replacement appeared to worsen the deficit and other environmental factors are difficult to quantitate. Thus, Tfm models demonstrate the importance of AR in mediating the positive effects of androgen to contribute to trabecular bone maintenance and in cortical bone, particularly at the periosteal surface [130].

p0410 Finally, a useful model for characterization of androgen signaling during development is represented by animals with tissue-selective modulation of AR expression, in which the effects of both overexpression and targeted deletion of AR have been characterized. Knockdown of genomic AR signaling in mature osteoblasts results in cancellous osteopenia, with increased bone resorption, a reduction in trabecular bone volume and a decrease in trabecular number, indicating the importance of AR signaling to maintain trabecular bone [136]. AR overexpression also results in a bone phenotype. Two distinct lines have been characterized with bone-targeted AR overexpression; one constructed with full-length AR under the control of the 3.6 kb type I collagen promoter and a second model employing the 2.3 kb type I collagen promoter to control AR overexpression. AR3.6-transgenic mice demonstrate overexpression in osteoblast stromal precursors and throughout the osteoblast lineage [27]. A major advantage of this model is overexpression of AR in the periosteal compartment, a known target for androgen anabolic action in the skeleton. The AR2.3-transgenic mice have overexpression of AR that is restricted to mature osteoblasts and osteocytes [104]. Since osteocytes are the most abundant cell type in bone [84] and also have the highest concentration of AR [83], these cells are likely an important target cell for androgen action and may represent a central mediator for skeletal responses to testosterone therapy in vivo. In general, AR overexpression in vivo results in a low turnover state in males with a significant reduction in cortical bone area due to inhibition of bone formation at the endosteal surface and a lack of marrow infilling. AR signaling also plays an important role in the trabecular bone, with increased trabecular bone volume via an increase in trabecular number but not width and reduced osteoclast number and/or activity. Finally, results indicate that enhanced androgen signaling in bone results in changes that are detrimental to biomechanical competence and whole bone strength, likely via reductions in osteoblast vigor and matrix quality. Again, there is little phenotype in female animals [133], so that the role of AR in normal female bone physiology is unclear.

It is instructive to compare and contrast the skeletal phenotypes that develop in the two distinct AR-transgenic lines.

(A)

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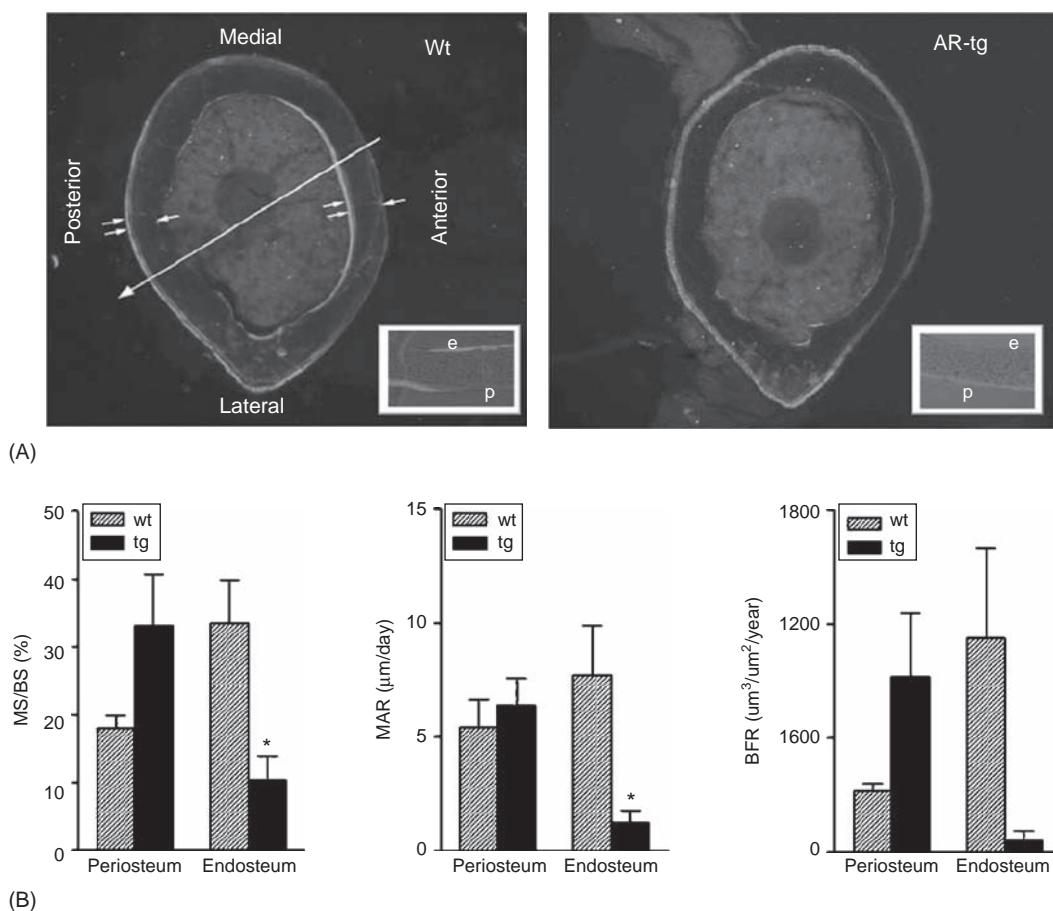
FIGURE 25.7   Characterization of cortical bone formation in AR-transgenic (AR-tg) mice. (A) Dynamic histomorphometric analysis was performed in cortical bone after fluorescent imaging microscopy in AR-tg males (n = 6–8) (see color plate section). (B) Mineralizing surface as a percent of bone surface (MS/BS), mineral apposition rate (MAR), bone formation rate (BFR) at both the endosteal and periosteal surfaces were determined in wildtype (wt) and AR-tg mice. *$P < 0.05$ [27]

In common between the two models, a phenotype with reduced bone turnover, reduced formation at endosteal surfaces, increased trabecular bone volume but compromised femoral strength in all biomechanical parameters tested is observed. With the exception of enhanced periosteal activity in AR3.6-transgenic males (Figure 25.7), neither model exhibits anabolic responses in the cortical bone compartment and, instead, both show inhibition of bone formation at the endocortical surface with compromised biomechanical properties and increased bone fragility. Thus, based on overlap in promoter activity, bone properties likely to be mediated at least in part by enhanced androgen signaling in mature osteoblasts/osteocytes include increased trabecular bone volume, reduced bone turnover, reduced formation with decreased osteoblast vigor at endosteal surfaces and compromised biomechanical strength with increased bone fragility. These results are observed in both models. The most striking contrast between the two AR-transgenic models is observed at periosteal surfaces in AR3.6-transgenic males, which show increased cortical bone formation in the periosteum and dramatic intramembranous calvarial thickening, likely

mediated by periosteal fibroblasts and/or immature osteoblasts. The specificity of the periosteal anabolic effect in AR3.6-transgenic males is consistent with previous reports documenting the importance of androgen signaling in periosteal expansion [137].

Periosteal bone formation defines the cross-sectional area of bone or bone width, whereas endosteal formation or resorption determines cortical thickness. Thus, androgen inhibition of medullary bone formation at the endosteal surface in males may subserve an important physiological adaptive function, being key for appropriate spatial distribution and maintenance of the total amount/weight of bone in the cortical envelope [104]. A reasonable hypothesis is that androgens strongly promote the addition of cortical width through periosteal growth, but balance that growth with inhibition in the marrow cavity so that the skeleton does not become too heavy [138]. Based on these findings, a model for the consequences of androgen signaling has been proposed, where the effects of AR activation are distinct in different skeletal compartments (Figure 25.8). Bone is also positively influenced by androgens at intramembranous

p0430
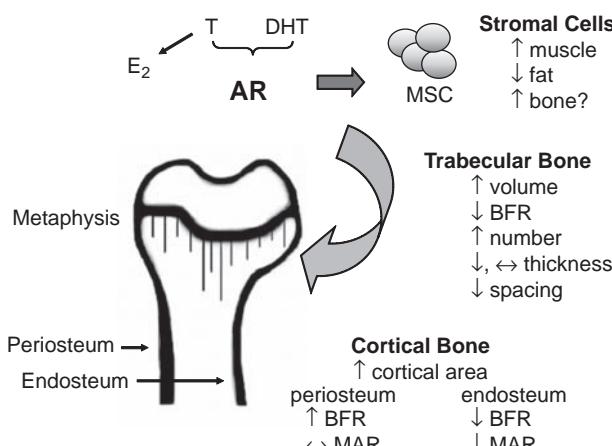
f0080   **FIGURE 25.8**   Model for androgen action in the skeleton mediated by AR transactivation. Androgen activation of AR influences a variety of target organs and skeletal sites, including marrow stromal cells and trabecular, cortical and intramembranous bone compartments. Arrows indicate the changes associated with androgen action. In trabecular bone, androgen action preserves or increases trabecular number, has little effect on trabecular thickness and, thus, reduces trabecular spacing. In cortical bone, AR activation results in reduced bone formation at the endosteal surface but stimulation of bone formation at the periosteal surface; correspondingly decreased periosteal but increased endosteal resorption results in no change in cortical area. In the transgenic models reviewed here, AR activation in mature bone cells in vivo results in a low turnover phenotype, with inhibition of bone formation and inhibition of gene expression in both osteoblasts and osteoclasts. In the absence of compensatory changes at the periosteal surface, these changes are detrimental to overall matrix quality, biomechanics and whole bone strength [104]

sites [27]. In addition, androgen administration increases muscle mass, partially mediated by effects on mesenchymal stem cell lineage commitment [77], likely to influence indirectly bone density through biomechanical linkage.

s0180   **EFFECTS ON THE PERIOSTEUM: THE ROLE OF AR VERSUS AROMATIZATION OF TESTOSTERONE**

p0440   As noted, androgen-mediated AR transactivation is likely a key determinant of the sexually dimorphic pattern of periosteal apposition that is most clearly demonstrated in male AR-transgenic mice in the absence of hormone administration [27]. Androgens are also unable to stimulate periosteal growth and radial bone expansion in the AR knockout model [139]. Essentially, all of the alterations induced by ORX (in both growing and mature animals) can be prevented, at least in part, by replacement with either testosterone or non-aromatizable androgens [5,140]. These results strongly suggest that aromatization of androgens to

estrogens cannot fully explain the actions of androgens on bone metabolism.

p0450   However, estrogens also seem play a role in the effects of androgen on periosteal apposition. Although AR activity is essential, low levels of estrogens are likely required for optimal stimulation of periosteal growth [139], as observed in aromatase deficiency in males [40]. Estrogens may also help prevent bone loss following castration in male animals. Vanderschueren et al. [141] reported that estradiol (and nandrolone) was capable of not only preventing the increase in biochemical indices stimulated by ORX, but also preventing cortical and trabecular bone loss. In fact, estradiol resulted in an absolute increase in trabecular bone volume not achieved with androgen replacement. Similarly, estrogen was reported to antagonize the increase in blood flow resulting from castration and to increase bone ash weight more consistently than testosterone. Although data thus far available are incomplete, these studies raise obvious questions of the overlap between the actions of androgens and estrogens in bone and/or the consequences of skeletal adaptation.

p0460   Although androgen and AR signaling is the likely mediator of periosteal expansion that results in larger bones in males, other hormones can influence periosteal growth and radial bone expansion, including growth hormone and PTH. While both testosterone and DHT stimulate periosteal bone formation in growth hormone receptor knockout male mice and do so without an effect on serum IGF-I or skeletal IGF-I expression, loss of growth hormone signaling dramatically reduced periosteal growth [137]. The ability of intermittent PTH to promote periosteal expansion may be mediated via enhanced differentiation of periosteal precursors [142]. Osteoblasts and periosteal fibroblasts frequently respond in a distinct fashion to hormonal, pharmacological or mechanical stimuli [143]. Interestingly, periosteal cell differentiation is much slower than osteoblasts derived from cancellous bone [144]. These results may not be surprising given that osteoblasts and periosteal cells reside in different bone niches and the source of periosteal fibroblasts remains an open question.

s0190   **EFFECTS ON BONE MASS IN ADULTS: EFFECTS OF CASTRATION IN YOUNG AND ADULT ANIMALS**

p0470   The effects of androgens on bone mass remodeling can be inferred by observing the results of androgen withdrawal after gonadectomy. In most studies, orchiectomy in young rats results in a reduction in cortical bone mass within 2–4 weeks. Calcium content of the femur or tibia [145], whole femoral, tibial or body bone mineral density [146,147] and tibial diaphyseal cortical area [5] have been shown to be lower in castrated than in sham operated controls. Similar

trends have been reported in young, castrate male mice [148]. In animals followed for longer periods after castration (90 days), the density of cortical bone was slightly (but not significantly) reduced, but bone area was clearly lessened in the diaphysis of the femur [145]. At least in part, the reduction in cortical bone mass appears to result from a reduction in periosteal bone formation rate induced by gonadectomy in males [4, 5]. This response is distinctly different than that induced by oophorectomy, which results in an increase in periosteal apposition in the period immediately after surgery. This divergent trend in the periosteal response to castration in male and female animals abolishes the sexual dimorphism usually present in radial bone growth. Endosteal bone formation does not seem to be affected by orchiectomy [4]. As another indication that the cortical skeleton is affected by androgens, the characteristic acute increase in creatine kinase activity induced from diaphyseal bone by androgen treatment is abolished by orchiectomy [140]. For unclear reasons, it remains intact in epiphyseal specimens. Although castration in the male tends to slow growth and weight gain, the effects on cortical bone histomorphometry are present in pair-fed rats and in groups in which there was no difference in growth rates [4, 5] indicating that the skeletal effects are not merely the indirect result of changes in body size or composition. Certainly, androgens are known to interact with the growth hormone-IGF system in the coordination of skeletal growth. Growth hormone deficiency in males has no net effect on endosteal growth but reduced by half expansion at the periosteal surface [149], underscoring the co-dependence of these two hormonal systems in the control of pubertal skeletal change.

p0480   Cancellous bone mass is also reduced in castrate young male rats. Tibial metaphyseal bone volume and vertebral bone mineral density are clearly reduced [4, 145, 147], an effect which is seen rapidly following castration [4]. The reduction in bone volume is dramatic, with differences between control and castrate of 40–50% appearing in 4–10 weeks [147, 150]. Rosen et al showed that measures of trabecular bone volume and mineral density diverged much more than did areal measures of the proximal tibia or distal femur (by dual energy X-ray absorptiometry) and speculated that this difference reflected a more intense bone deficit from trabecular than from cortical compartments [147]. An important issue that remains unresolved is whether the bone deficit is a result of actual loss of bone mass following castration, or whether the differences between castrate and control animals results from a failure of castrate animals to accrue bone normally. Nevertheless, bone changes following orchiectomy occur in the presence of an increase in skeletal blood flow, osteoclast numbers and surface [4], serum and urine calcium levels [4] and increased serum tartrate resistant acid phosphatase activity [146]. All these findings strongly suggest an increase in bone remodeling and bone resorption. On the other hand, in one report, distal

femoral bone loss following castration is accompanied by a reduction in bone remodeling [151]. Parathyroid hormone concentrations have not been measured in these experiments, but vitamin D concentrations do not appear to be altered by orchiectomy [4]. In sum, trabecular bone mass, as well as cortical mass, is clearly dependent on adequate androgen action in the growing male animal.

p0490   Results from animal studies also support an effect of androgen on bone formation in the mature animal. In mature rats, castration has been used to evaluate the consequences of loss of sex steroids (both estrogen and androgen). It is well established that castration eventually results in osteopenia and both cortical and trabecular compartments are affected. At a time when longitudinal growth has slowed markedly, pronounced differences as a consequence of castration appear in cortical bone ash weight per unit length, cross-sectional area, cortical thickness and bone mineral density [141,152]. Castration results in changes in both trabecular and cortical bone compartments and dramatic bone loss in trabecular bone is noted in both males and females, but sex-specific responses are most dimorphic in cortical bone. For example, distinct effects of androgen are seen with gonadectomy when comparing the effects of ORX in male versus ovariectomy (OVX) in female rats. OVX and the associated loss of sex steroids in the female generally result in decreased trabecular area with increased osteoclast number. In cortical bone, an increase at the periosteal surface is seen with circumferential enlargement but a decrease in endosteal labeling. These results demonstrate that estrogen protects trabecular bone predominantly through inhibition of osteoclast activity/recruitment, but has an inhibitory action at the periosteal surface as noted above [130]. In the male, ORX with the attendant loss of sex steroids also result in decreased trabecular area with increased osteoclast number but, in contrast with the female, periosteal formation in cortical bone is reduced with the loss of androgen. Androgen treatment is effective in suppressing the acceleration of bone remodeling normally seen after ORX [153]. This divergent trend in the periosteal response to castration in male and female animals abolishes the sexual dimorphism usually present in radial bone growth. In the intact animal, the stimulation of endosteal formation by estrogen compensates for the lack of periosteal formation, thus leading to no difference in cortical width or biomechanical strength between the sexes. Nevertheless, factors that influence periosteal apposition may constitute an important therapeutic class since periosteal bone formation is often a neglected determinant of bone strength [154]. ORX shows little net effect on the endosteal surface in males [149] or slight reductions likely due to increased resorption. Consistent with this, increased intracortical resorption cavities are reported to result from ORX [155]. As might be expected in light of these changes, breaking strength (N) is decreased in cortical bone [149]. In addition, it appears that ORX affects cranial development more than

OVX [156], suggesting that androgen action is also important in intramembraneous bone.

p0500   In addition to changes in bone size at the periosteal surface, trabecular bone volume is reduced rapidly after castration as well [157,158] and osteopenia becomes pronounced with time [159]. This bone loss appears to result in part from increased bone resorption, as it is associated with increased resorption cavities, osteoclasts and blood flow [160]. Dynamic histomorphometric and biochemical measures of bone remodeling increase quickly [145,158], with evidence of increased osteoclast numbers only 1 week after castration [158]. These changes include an increase in osteoblastic activity as well as increased bone resorption, reflecting an initial high turnover state that is followed by a reduction in remodeling rates and low turnover osteopenia. In the SAMP6 mouse, a model of accelerated senescence in which osteoblastic function is impaired, the rise in remodeling following ORX is blunted, which has been interpreted as evidence that the early changes after gonadectomy are dependent on osteoblast-derived signals [161]. As noted above, androgens reduce osteoclast formation and activity [105], which may be partially mediated by increased OPG levels [27,108]. The initial phase of increased bone remodeling activity subsides with time [145,160] and, by 4 months, there is evidence of a depression in bone turnover rates in some skeletal areas [160]. As in younger animals, indices of mineral metabolism are not altered by these changes in skeletal metabolism [141]. Careful histomorphometric analysis by Ohlsson and workers of androgen action in ORX mice has shown that the bone sparing effect of AR activation in trabecular bone is distinct from the bone-sparing effect of ERα at that site [162]. The analysis demonstrated that AR activation does preserve the number of trabeculae, but does not preserve thickness or volumetric density, nor mechanical strength in cortical bone.

p0510   As a potential model for the effects of hypogonadism in humans, animal models therefore suggest an early phase of high bone turnover and bone loss after ORX, followed by a reduction in remodeling rates and osteopenia. The remodeling imbalance responsible for loss of bone mass appears complex, as there are changes in rates of both bone formation and resorption and patterns that vary from one skeletal compartment to another. These overall changes are similar in overall pattern to those noted in female animals after castration, in which a loss of estrogen signaling has been associated with a stimulation of osteoblast progenitor differentiation, an increase in osteoclast numbers, bone resorption and bone loss [163].

s0200   **ANDROGENS IN THE FEMALE ANIMAL**

Of course androgens are present in females as well as males and may affect bone metabolism. In castrate female rats,

DHT (a non-aromatizable androgen) administration suppresses elevated concentrations of bone resorption markers, as well as those of increased osteocalcin [164]. However, alkaline phosphatase activity increases further. Additional evidence to support the contention that androgens play a role in females includes the fact that antiandrogens (e.g. flutamide) are capable of evoking osteopenia in intact (i.e. fully estrogenized) female rats [6]. This obviously suggests that androgens provide crucial support to bone mass independent of estrogens in the adult. Of interest, the character of the bone loss induced by flutamide suggested that estrogen prevents bone resorption, whereas androgens may stimulate bone formation. In periosteal bone, DHT and testosterone appear to stimulate bone formation after ORX in young male rats, whereas in castrate females they suppress bone formation [5], perhaps reflecting an interaction or synergism between sex steroids and their effects on bone. As noted above, combination therapy with estrogen and androgen in postmenopausal women is more beneficial than either steroid alone [8–10], which has been confirmed in an animal model [165]. There is also some information concerning androgens in additional animal models, including primates. For instance, in adult female cynomolgus monkeys, testosterone treatment increased cortical and trabecular bone density as well as biomechanical strength [166].

EFFECTS OF REPLACEMENT SEX STEROIDS AFTER CASTRATION   s0210

p0530   Essentially, all of the alterations induced by orchiectomy (in both growing and mature animals) can be prevented by replacement with either testosterone or non-aromatizable androgens [5,140,150,167,168]. These results strongly suggest that aromatization of androgens to estrogens cannot fully explain the actions of androgens on bone metabolism.

p0540   On the other hand, estrogens also seem to prevent bone loss following castration in male animals. Vanderscheuren et al reported that estradiol (plus nandrolone) was capable of not only preventing the increase in biochemical indices stimulated by orchiectomy, but was also able to prevent cortical and cancellous bone loss [141]. In fact, estradiol resulted in an absolute increase in trabecular bone volume not achieved with androgen replacement. Similarly, estrogen was reported to antagonize the increase in blood flow resulting from castration and to increase bone ash weight more consistently than testosterone. Although the data thus far available are incomplete, these studies raise obvious questions of the overlap between the actions of androgens and estrogens in bone.

p0550   The gender reverse condition of employing androgen replacement in female animals is also instructive. Non-aromatizable androgens are capable of preventing or reversing osteopenia and abnormalities in bone remodeling in   p0520

oophorectomized females [5]. These actions apparently result from the suppression of trabecular bone resorption as well as stimulation of periosteal bone formation [169]. Very similar results have been reported following the treatment of oophorectomized animals with DHEA [170]. Moreover, blockage of androgen action with an AR antagonist in female rats already treated with an estrogen antagonist increases bone loss and indices of osteoclast activity more than treatment with an estrogen antagonist alone [171], again indicating that ovarian androgens (apart from estrogens) exert a protective effect on bone in females. Analogously, androstenedione reduces (although does not abrogate) trabecular bone loss (and remodeling alterations) in oophorectomized animals treated with an aromatase inhibitor [172]. This protective effect was blocked by the addition of an AR antagonist [173]. Finally, whereas aromatase inhibition in male rats reduces bone mass, the large increase in remodeling induced by ORX does not occur in these animals [41]. Also, ORX in ERKO mice further reduces bone mass [73]. The latter observation implicates a role for androgens in the maintenance of bone mass in ERKO mice.

SUMMARY

s0220

p0560 The effects of androgens on bone health are obviously pervasive and complex. Androgens are important in the maintenance of a healthy skeleton and influence skeletal modeling and remolding by multiple mechanisms through effects on osteoblasts, osteocytes, osteoclasts and even perhaps an influence on the differentiation of pluripotent stem cells toward distinct lineages. The specific effects of androgens on bone cells are mediated directly through an androgen receptor- (AR-) signaling pathway, but there are also indirect contributions to overall skeletal health through aromatization and estrogen receptor (ER) signaling. The effects of androgens are particularly dramatic during growth in boys and may play a role during this period in girls as well. Androgens strongly promote the addition of cortical width through periosteal growth but balance that growth with inhibition of formation in the marrow cavity so that the skeleton does not become too heavy during development and, thus, may subserve an important physiological adaptive function. Throughout the rest of life, androgens can affect skeletal function in both sexes. Since the effects of androgens are still poorly characterized, more needs to be done to unravel the mechanisms by which androgens influence the physiology and pathophysiology of bone and there remains much to be learned about the roles of androgens at all levels in both males and in females. The interaction of androgens and estrogens and how their respective actions can be utilized for specific diagnostic and therapeutic benefit are important but unanswered issues. With an increase in the understanding of the nature of androgen effects will come greater opportunities to use their positive actions in the prevention and treatment of a wide variety of skeletal disorders.

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AUTHOR QUERY

{AUQ1}There's another reference (Lorentzen JBMR 2006, I think, that looks at T and E and structure in young men and reports that T is associated with larger bones an la arger medullary space, and E with smaller bones an smaller medullary space). This goes along nicely with your comments at the end of the AR transgenics section below.
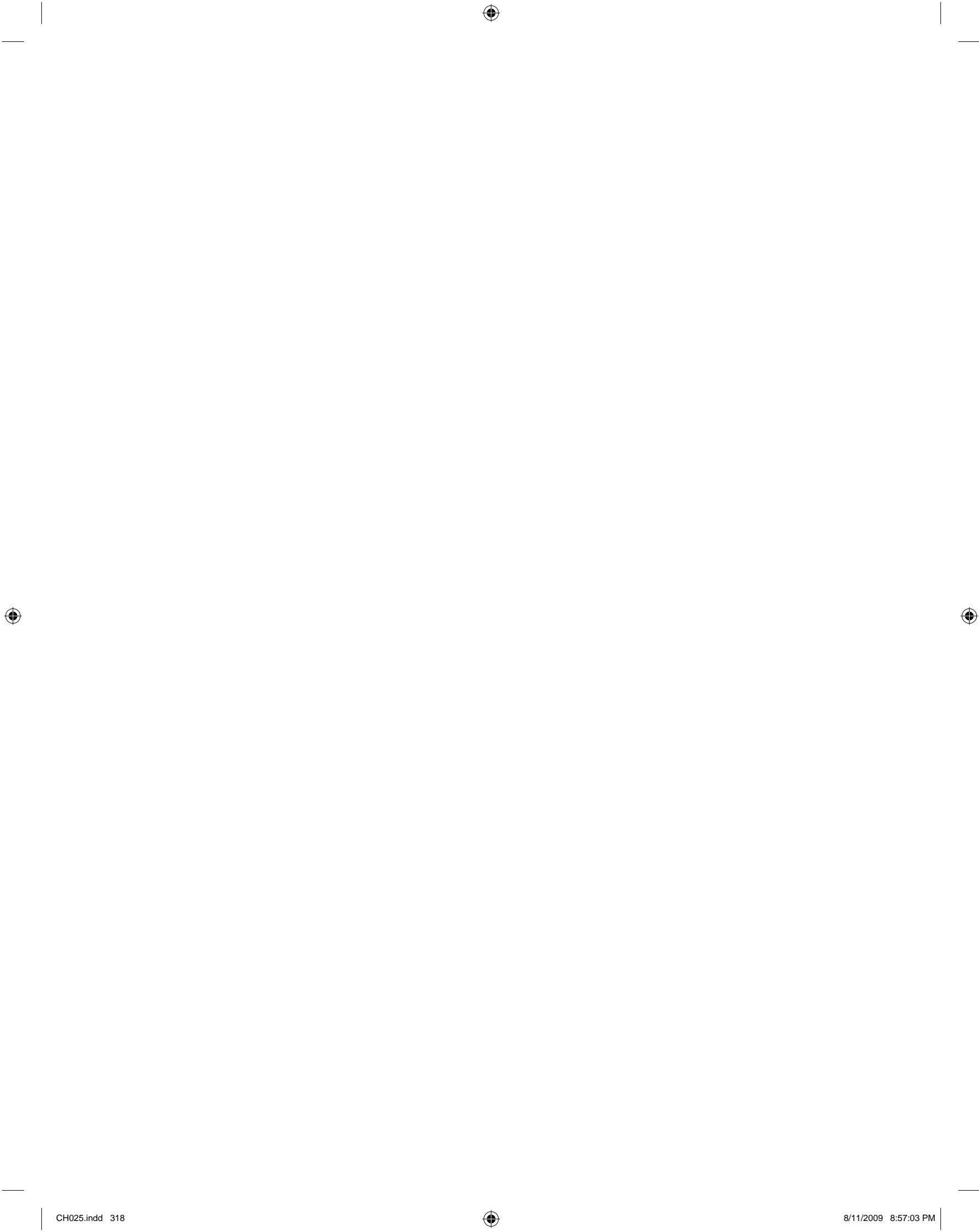
Androgen and Skeletal Biology: Basic Mechanisms

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Abstract

Because there remains confusion interpreting the skeletal actions of sex steroids, the specific mechanisms by which androgens affect skeletal homeostasis are becoming the focus of intensified research. As a classic steroid hormone, the biological cellular signaling responses to androgen are mediated through the androgen receptor (AR), a ligand-inducible transcription factor. ARs have been identified in a variety of cells found in bone. Characterization of AR expression in these cells thus clearly identifies bone as a target tissue for androgen action. The direct effects of androgen that influence the complex processes of proliferation, differentiation, mineralization and gene expression in the osteoblast are being characterized, but much remains to be established. Androgen effects on bone may also be indirectly modulated and/or mediated by other autocrine and paracrine factors in the bone microenvironment. This chapter will review recent progress on the characterization of molecular and cellular mechanisms that underlie androgen action in bone.

1. Introduction

The obvious impact of the menopause on skeletal health has focused much of the research describing the general action of gonadal steroids on the specific effects of estrogen in bone. However, androgens clearly have important beneficial effects, in both men and women, on skeletal development and on the maintenance of bone mass (1, 2). Thus it has been demonstrated that androgens (a) influence growth plate maturation and closure helping to determine longitudinal bone growth during development, (b) mediate regulation of trabecular (cancellous) and cortical bone mass in a fashion distinct from estrogen, leading to a sexually dimorphic skeleton, (c) modulate peak bone mass acquisition, and (d) inhibit bone loss (2). In castrate animals, replacement with nonaromatizable androgens (*e.g.* 5α-dihydrotestosterone, DHT) yields beneficial effects that are clearly distinct from those observed with estrogen replacement (3, 4). In intact females, blockade of the androgen receptor (AR) with the specific AR antagonist hydroxyflutamide results in osteopenia (5). Furthermore, treatment with nonaromatizable androgen alone in females results in improvements in bone mineral density (6). Finally, combination therapy with estrogen and androgen in post-menopausal women is more beneficial than either steroid alone (7-9), indicating non-parallel and distinct pathways of action. Combined, these reports illustrate the distinct actions of androgens and estrogens on the skeleton. Thus, in both men and women it is probable that androgens and estrogens each have important yet distinct functions during bone development, and in the subsequent maintenance of skeletal homeostasis in the adult. With the awakening awareness of the importance of the effects of androgen on skeletal homeostasis, and the potential to make use of this information for the treatment of bone disorders, much remains to be learned.

2. Molecular mechanisms of androgen action in bone cells: the androgen receptor (AR)

Direct characterization of AR expression in a variety of tissues, including bone (10), was made possible by the cloning of the AR cDNA (11, 12). The AR is a member of the class I (so-called classical or steroid) nuclear receptor superfamily, as are the (estrogen receptor) ERα and ERß isoforms, the progesterone receptor, the mineralocorticoid and glucocorticoid receptor (13). Steroid receptors are transcription factors with a highly-conserved modular design characterized by three functional domains: the transactivation, DNA binding and ligand binding domains. In the absence of ligand, the AR protein is generally localized in the cytoplasmic compartment of target cells in a large complex of molecular chaperones, consisting of loosely bound heat-shock, cyclophilin and other accessory proteins (14). Interestingly, in the unliganded form, AR conformation is unique with a relatively unstructured amino-terminal transactivation domain (15). As lipids, androgens can freely diffuse through the plasma membrane to bind the AR to induce a conformational change. Once bound by ligand, the AR dissociates from the multiprotein complex, translocates to the nucleus and recruits coactivators or corepressors that can display cell-type specific expression (16), allowing the formation of homodimers (or potentially heterodimers) that activate a cascade of events in the nucleus (17). Bound to DNA, the AR influences transcription and/or translation of a specific network of genes, leading to the cellular response to the steroid.

2.1 The androgen receptor signaling pathway

Once bound by ligand, the AR is activated. As shown in **Figure 1,** this allows the formation of homodimers (or potentially heterodimers) that bind to DNA at palindromic androgen response elements (AREs) in androgen responsive gene promoters. Classic ARE sequences are found in the proximal promoter as a motif represented by an inverted repeat

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separated by 3 bp (18) similar to glucocorticoid response elements (19). However, our understanding of hormone binding sites in DNA are becoming better characterized and are more complex than originally described (20). Thus, AR binding sites that influence expression, both positively and negatively, are likely distributed throughout the genome with sequences more complex and diverse than simple ARE repeats. DNA binding of the activated AR organizes a cascade of events in the nucleus leading to transcription and translation of a specific network of genes that is responsible for the cellular response to the steroid (17). In the classic model of steroid action, the latent receptor is converted into a transcriptionally active form by simple ligand binding. Again, this model is now considered an over-simplification, with the understanding that signaling pathways and additional proteins (for example, coactivators or corepressors as described below and shown in **Figure 1**) within the cell can influence steroid receptor transduction activity. Furthermore, post-translational modification of the receptor by acetylation, phosphorylation and/or ubiquitination can occur (21). For example**,** steroid receptor phosphorylation can result from signal transduction cascades initiated at the cell membrane, such as from activation of src kinases by growth factors (22). It has been shown that steroid receptor phosphorylation can lead to alterations of the responsiveness of steroid receptors to cognate ligands or, in some cases, even result in ligand-independent activation.

Such potential modification(s) of AR action in bone cells is only poorly characterized; whether the AR in osteoblasts undergoes post-translational processing that might influence AR activity as described in other tissues (23, 24), and the potential functional implications (25, 26), are also unknown. Ligand-independent activation of AR has also been described in other tissues (27), but has not been explored in bone. AR activity may also be influenced by receptor modulators, such as the nuclear receptor coactivators or corepressors (22, 28, 29).

These coactivators/corepressors can influence the downstream signaling of nuclear receptors through multiple mechanisms, including histone acetylation/deacetylation, respectively, that results in chromatin remodeling. Such activities may reflect both the cellular context and the particular promoter involved. AR specific coactivators have been identified (30), many of which interact with the ligand binding domain of the receptor (31). Expression and regulation of these modulators may thus influence the ability of steroid receptors to regulate gene expression in bone (18), but this remains underexplored with respect to androgen action. The specific coactivator/corepressor profile present in cells representing different bone compartments (*i.e.*, periosteal cells, proliferating or mineralizing cells) may help determine the activity of selective AR modulators (SARMS) as described below, that influence transcriptional activity of the AR.

The number of specific androgen binding sites in osteoblasts varies, depending on methodology and the cell source, from 1,000-14,000 sites/cell (32-35), but is in a range seen in other androgen target tissues. Furthermore, the binding affinity of the AR found in osteoblastic cells (K_d = 0.5-2 x 10^{-9}) is typical of that found in other tissues. Androgen binding is specific, without significant competition by estrogen, progesterone or dexamethasone (33, 35, 36). Finally, testosterone and DHT appear to have relatively similar binding affinities (33, 37). All these data are consistent with the notion that the direct biologic effects of androgenic steroids in osteoblasts are mediated at least in part via classic mechanisms associated with the AR.

In addition to the classical AR present in bone cells, several other androgen-dependent signaling pathways have been described. Specific binding sites for weaker adrenal androgens (such as dehydroepiandrosterone, DHEA) have been described (38); DHEA can also transactivate AR (39), thus raising the possibility that DHEA or similar androgenic compounds may also have direct effects in bone. DHEA and its metabolites may

also bind and activate additional receptors, including ER, peroxisome proliferator activated

receptor-α and pregnane X receptor (40). Bodine *et al.* (41) showed that DHEA caused a

rapid inhibition of c-*fos* expression in human osteoblastic cells that was more robust than

seen with the classical androgens (DHT, testosterone, androstenedione). In addition, DHEA

may inhibit bone resorption by osteoclasts when in the presence of osteoblasts, likely

through changes in osteoprotgerin (OPG) and receptor activator of NFκB ligand (RANKL)

concentrations (42). AR may also interact with other transcription factors, such as NF-κB,

CREB-binding protein and different forms of AP-1, to generally repress transcription without

DNA binding. Alternatively, androgens may be specifically bound in osteoblastic cells by a

novel 63-kDa cytosolic protein (43). In addition, there are reports of distinct AR

polymorphisms identified in different races that may have biological impact on androgen

responses (44), but to date none have an effect with respect to bone tissue (45). These

different isoforms have the potential to interact in distinct fashions with other signaling

molecules, such as c-Jun (46). Finally, androgens may regulate osteoblast activity via rapid

nongenomic mechanisms (47, 48) through membrane receptors displayed at the bone cell

surface (49). The role and biologic significance of these non-classical signaling pathways in

androgen-mediated responses in bone remains controversial, and most data suggests that

genomic signaling may be the more significant regulator in bone and other tissues (50-53).

2.2 Localization of androgen receptor expression

　　　Clues about the potential sequelae of AR signaling may be derived from a better

understanding of the cell types in which receptor expression is documented. In the bone

microenvironment, the localization of AR expression in osteoblasts has been described in

intact human bone by using immunocytochemical techniques (10, 54). In developing bone

from young adults, Abu *et al.* (10) showed ARs were predominantly expressed in active osteoblasts at sites of bone formation (**Figure 2**). ARs were also observed in osteocytes embedded in the bone matrix. Importantly, both the pattern of AR distribution and the level of expression were similar in males and in females. In addition, expression of the AR has been characterized in cultured osteoblastic cell populations isolated from bone biopsy specimens, determined at both the mRNA level and by binding analysis (35). Expression varied according to the skeletal site of origin and age of the donor of the cultured osteoblastic cells: AR expression was higher at cortical and intramembranous bone sites, and lower in trabecular bone. This distribution pattern correlates with androgen-responsiveness in the bone compartment. AR expression was highest in osteoblastic cultures generated from young adults and somewhat lower in samples from either prepubertal or senescent bone. Again, no differences were found between male and female samples, suggesting that differences in receptor number *per se* do not underlie development of a sexually dimorphic skeleton. Interestingly, ARs are also expressed in bone marrow stromal (55) and mesenchymal precursor cells (56), pluripotent cells that can differentiate into muscle, bone and fat. Androgen action may modulate precursor differentiation toward the osteoblast and/or myoblast lineage, while inhibiting differentiation toward the adipocyte lineage (57). These effects on stromal differentiation could underlie some of the well-described consequences of androgen administration on body composition including increased muscle mass (58). To date, it has not been established how significant the contribution is, of the increased muscle mass associated with androgen administration, to positively influence bone quality. Bone marrow stomal cells are also responsive to sex steroids during the regulation of osteoclastogenesis.

Because androgens are so important in bone development at the time of puberty, it is not surprising that ARs are also present in epiphyseal chondrocytes (10, 59). Noble *et al.* (54) described AR expression mainly in the narrow zone of proliferating chondrocytes in the growth plate, with reduced expression in hypertrophied cells. The expression of ARs in such a wide variety of cell types known to be important for bone modeling during development, and remodeling in the adult, provides strong evidence for direct actions of androgens in bone and cartilage tissue. These results also presage the complexity of androgen effects on developing bone tissue.

Potential modulation of osteoclast action by androgen is suggested by reports of AR expression in the osteoclast (60). Androgen treatment reduces bone resorption of isolated osteoclasts (61), inhibits osteoclast formation (62) including formation stimulated by parathyroid hormone (PTH) (63), and may play a direct role regulating aspects of osteoclast activity based on results in AR null mice (64). Indirect effects of androgen to modulate osteoclasts via osteoblasts are indicated by the increase in osteoprotegerin (OPG) levels following testosterone treatment in osteoblasts (65) and increased OPG serum concentrations in skeletally-targeted AR-transgenic male mice (66). In addition, DHEA treatment has been shown to increase the OPG/RANKL ratio in osteoblastic cells and to inhibit osteoclast activity in coculture (67). Although androgen may be a less significant determinant of bone resorption *in vivo* than estrogen (68, 69), this remains controversial (70).

2.3 Regulation of androgen receptor expression

The regulation of AR expression in osteoblasts is incompletely understood. Homologous regulation of AR mRNA by androgen has been described that is tissue specific; up-regulation by androgen exposure is seen in a variety of mesenchymal cells including

osteoblasts (71-74) whereas in prostate and smooth muscle tissue, down-regulation is observed after androgen exposure (73, 75) (**Figure 3**). The androgen mediated up-regulation observed in osteoblasts occurs, at least in part, through changes in AR gene transcription (73, 74). No effect, or even inhibition, of AR mRNA by androgen exposure in other osteoblastic models has also been described (35, 76). Interestingly, a novel property of the AR is that binding of androgen increases AR protein levels, that has been shown in osteoblastic cells as well (74). This property distinguishes AR from most other steroid receptor molecules that are down-regulated by ligand binding. The elevated AR protein levels may be a consequence of increased stability mediated by androgen binding, resulting from N-terminal and C-terminal interactions (77), but the stability of AR protein in osteoblastic cells has not been determined to date. The mechanism(s) that underlie tissue specificity in autologous AR regulation, and the possible biological significance, is not yet understood. It is possible that AR up-regulation by androgen in bone may result in an enhancement of androgen responsiveness at times when androgen levels are rising or elevated.

Quantitative determination of the level of receptor expression during osteoblast differentiation is difficult to achieve in bone slices. However, analysis of AR, ERα and ERß mRNA and protein expression during osteoblast differentiation *in vitro* demonstrates that each receptor displays distinct differentiation-stage expression patterns in osteoblasts (**Figure 4**) (78). The levels of AR expression increase throughout osteoblast differentiation with the highest AR levels seen in mature osteoblast/osteocytic cultures. These results suggest that an important compartment for androgen action may be mature, mineralizing osteoblasts, and indicate that osteoblast differentiation and steroid receptor regulation are intimately associated. Given that the osteocyte is the most abundant cell type in bone, and a likely mediator of focal bone deposition and response to mechanical strain (79), it is not

surprising that androgens may also augment the osteo-anabolic effects of mechanical strain in osteoblasts (80).

3. Effects of androgens on osteoblastic cells

Evidence suggests that androgens act directly on the osteoblast and there are reports, some in clonal osteoblastic cell lines, of modulatory effects of gonadal androgen treatment on proliferation, differentiation, matrix production and on mineral accumulation (81). Not surprisingly, androgen has been shown to influence bone cells in a complex fashion.

3.1 Androgens and osteoblast proliferation

As an example, the effect of androgen on osteoblast proliferation has been shown to be biphasic in nature, with enhancement following short or transient treatment but significant inhibition following longer treatment. As a case in point, Kasperk *et al* (82, 83) demonstrated in osteoblast-like cells in primary culture (murine, passaged human) that a variety of androgens in serum-free medium increase DNA synthesis ([^{3}H]thymidine incorporation) and cell counts. Testosterone and nonaromatizable androgens (DHT and fluoxymesterone) were nearly equally effective regulators. Yet the same group (84) reported that prolonged DHT treatment inhibited normal human osteoblastic cell proliferation (cell counts) in cultures pretreated with DHT. Hofbauer *et al* (85) examined the effect of DHT exposure on proliferation in hFOB/AR-6, an immortalized human osteoblastic cell line stably transfected with an AR expression construct (with ~4,000 receptors/cell). In this line, DHT treatment inhibited cell proliferation by 20-35%. Consistent with stimulation, Somjen *et al* have demonstrated increased creatine kinase specific activity in male osteoblastic cells after exposure to DHT for 24 hours (86). Although these various studies employed different model

systems and culture conditions, it appears exposure time is an important variable. Clear time dependence for the response to androgen has been shown by Wiren *et al* (87), where osteoblast proliferation was stimulated at early treatment times, but with more prolonged DHT treatment osteoblast viability decreased (**Figure 5**). This result was AR dependent (inhibitable by coincubation with flutamide), and was observed in both normal rat calvarial osteoblasts and in AR stably transfected MC-3T3 cells. In mechanistic terms, reduced viability was associated with overall reduction in mitogen-activated (MAP) kinase signaling and with inhibition of *elk-1* gene expression, protein abundance and extent of phosphorylation. The inhibition of MAP kinase activity after chronic androgen treatment again contrasts with stimulation of MAP kinase signaling and AP-1 transactivation observed with brief androgen exposure (87), that may be mediated through non-genomic mechanisms (47, 88, 89).

3.2 Androgens and osteoblast apoptosis

As a component of control of osteoblast survival, it is also important to consider the process of programmed cell death, or apoptosis (90). In particular, as the osteoblast population differentiates *in vitro*, the mature bone cell phenotype undergoes apoptosis (91). With respect to the effects of androgen exposure, chronic DHT treatment has been shown to result in enhanced osteoblast apoptosis in both proliferating osteoblastic (at day 5) and in mature osteocytic cultures (day 29) (92). In this report, inhibition observed with DHT treatment was opposite to inhibitory effects on apoptosis seen with E_2 treatment (**Figure 6**). An androgen-mediated increase in the Bax/Bcl-2 ratio was also observed, predominantly through inhibition of Bcl-2 and was dependent on functional AR. Overexpression of *bcl-2* or RNAi knockdown of *bax* abrogated the effects of DHT, indicating that increased Bax/Bcl-2

was necessary and sufficient for androgen-enhanced apoptosis. The increase in the Bax/Bcl-2 ratio was at least in part a consequence of reductions in Bcl-2 phosphorylation and protein stability, consistent with inhibition of MAP kinase pathway activation after DHT treatment as noted above. *In vivo* analysis of calvaria in AR-transgenic male mice demonstrated enhanced TUNEL staining in both osteoblasts and osteocytes, and was observed even in areas of new bone growth (92). This may not be surprising, given an association between new bone growth and apoptosis (93), as has been observed in other remodeling tissues and/or associated with development and tissue homeostasis (94). Apoptotic cell death could thus be important in making room for new bone formation and matrix deposition, which may have clinical significance by influencing bone homeostasis and bone mineral density (95). Thus, mounting evidence suggests that chronic androgen treatment does not increase osteoblast number or viability in the mature bone compartment. It is interesting to speculate that the inhibitory action of androgens in osteoblasts at the endosteal surface is important for the relative maintenance of cortical width (which is similar between males and females), given the strong stimulation at the periosteal surface, such that the skeleton does not become excessively large and/or heavy during development.

3.3 Effects of androgens on the differentiation of osteoblastic cells

Osteoblast differentiation is often characterized by changes in alkaline phosphatase activity and/or alterations in the expression of important extracellular matrix proteins, such as type I collagen, osteocalcin, and osteonectin. Enhanced osteoblast differentiation, as measured by increased matrix production, has been shown to result from androgen exposure. Androgen treatment in both normal osteoblasts and transformed clonal human osteoblastic cells (TE-89) appears to increase the proportion of cells expressing alkaline phosphatase activity, thus representing a shift toward a more differentiated phenotype (82). Kasperk *et al.*

subsequently reported dose-dependent increases in alkaline phosphatase activity in both high and low-alkaline phosphatase subclones of SaOS2 cells (96) and human osteoblastic cells (84). However, there are also reports in a variety of model systems of androgens either inhibiting (85) or having no effect on alkaline phosphatase activity (71, 97), which may reflect both the complexity and dynamics of osteoblastic differentiation. There are also reports of androgen-mediated increases in type I α-1 collagen protein and mRNA levels (37, 96-98) in certain circumstances, and increased osteocalcin mRNA or protein secretion (84, 98). Consistent with increased collagen production, androgen treatment has also been shown to stimulate mineral accumulation in a time and dose-dependent manner (71, 84, 99). However, transgenic mice with targeted overexpression of AR in the osteoblast lineage showed decreased levels of most bone markers *in vivo* in total RNA extracts derived from long bone samples, including decreased collagen, osterix and osteocalcin gene expression (66). These results suggest that, under certain conditions, androgens may enhance osteoblast differentiation and could thus play an important role in the regulation of bone matrix production and/or organization. On the other hand, many positive anabolic effects of androgen may be limited to distinct osteoblastic populations, for example in the periosteal compartment (2, 66).

3.4 Interaction with other factors to modulate bone formation and resorption

The effects of androgens on osteoblast activity must certainly also be considered in the context of the very complex endocrine, paracrine and autocrine milieu in the bone microenvironment. Systemic and/or local factors can act in concert, or can antagonize, to influence bone cell function. This has been well described with regard to modulation of the effects of estrogen on bone (see for example 100, 101, 102). Androgens have also been shown to regulate well-known modulators of osteoblast proliferation or function. The most

extensively characterized growth factor influenced by androgen exposure is transforming growth factor-ß (TGF-ß). TGF-ß is stored in bone (the largest reservoir for TGF-ß) in a latent form and has been shown to be a mitogen for osteoblasts. Androgen treatment can increase TGF-ß activity in osteoblastic cultures: the expression of some TGF-ß mRNA transcripts (apparently TGF-ß2) were increased but no effect on TGF-ß1 mRNA abundance was observed (41, 83), but also see (103). At the protein level, specific immunoprecipitation analysis reveals DHT mediated increases in TGF-ß activity to be predominantly TGF-ß2 (41, 84). DHT has also been shown to inhibit both TGF-ß gene expression and TGF-ß-induced early gene expression that correlates with growth inhibition in this cell line (85). The TGF-ß-induced early gene has been shown to be a transcription factor that may mediate some TGF-ß effects (104). These results are consistent with the notion that TGF-ß may mediate androgen effects on osteoblast proliferation. On the other hand, TGF-ß1 mRNA levels are increased by androgen treatment in human clonal osteoblastic cells (TE-89), under conditions where osteoblast proliferation is slowed (37). Thus, the specific TGF-ß isoform may determine osteoblast responses. It is interesting to note that *in vivo*, orchiectomy (ORX) drastically reduces bone content of TGF-ß levels, and testosterone replacement prevents this reduction (105). These data support the findings that androgens influence cellular expression of TGF-ß, and suggest that the bone loss associated with castration is related to a reduction in growth factor abundance induced by androgen deficiency.

Other growth factor systems may also be influenced by androgens. Conditioned media from DHT treated normal osteoblast cultures are mitogenic, and DHT pretreatment increases the mitogenic response to fibroblast growth factor and to insulin like growth factor II (IGF-II) (83). In part, this may be due to slight increases in IGF-II binding in DHT treated cells (83), as IGF-I and IGF-II levels in osteoblast conditioned media are not affected by androgen (83, 106). Although most studies have not found regulation of IGF-I or IGF-II

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abundance by androgen exposure (33, 83, 106), there is a report that IGF-I mRNA levels are significantly up-regulated by DHT (107). Androgens may also modulate expression of components of the AP-1 transcription factor (41) or AP-1 transcriptional activation (87). Thus, androgens may modulate osteoblast differentiation via a mechanism whereby growth factors or other mediators of differentiation are regulated by androgen exposure.

Androgens may modulate responses to other important osteotropic hormones/regulators. Testosterone and DHT specifically inhibit the cAMP response elicited by PTH or parathyroid hormone-related protein (PTHrP) in the human clonal osteoblast-like cell line SaOS-2, while the inactive or weakly active androgen 17α-epitestosterone had no effect, via an effect on effector G_s-adenylyl cyclase (108-110). The production of prostaglandin E_2 (PGE_2), another important regulator of bone metabolism, is also affected by androgens. Pilbeam and Raisz showed that androgens (both DHT and testosterone) were potent inhibitors of both parathyroid hormone and interleukin-1 stimulated PGE_2 production in cultured neonatal mouse calvaria (111). The effects of androgens on parathyroid hormone action and PGE_2 production suggest that androgens could act to modulate (reduce) bone turnover in response to these agents.

Finally, both androgen (112) and estrogen (101, 113) can inhibit production of interleukin-6 by osteoblastic cells (but see 114). In stromal cells of the bone marrow, androgens have been shown to have potent inhibitory effects on the production of interleukin-6 and the subsequent stimulation of osteoclastogenesis by marrow osteoclast precursors (115). Interestingly, adrenal androgens (androstenediol, androstenedione, DHEA) have similar inhibitory activities on interleukin-6 gene expression and protein production by stromal cells (115). Moreover, androgens inhibit the expression of the genes encoding the two subunits of the IL-6 receptor (gp80 and gp130) in the murine bone marrow, another mechanism which may blunt the effects of this osteoclastogenic cytokine in intact

animals (116). In these aspects, the effects of androgens seem to be very similar to those of estrogen, which may also inhibit osteoclastogenesis via mechanisms that involve interleukin-6 inhibition and/or OPG/RANKL ratio changes.

4. Metabolism of androgens in bone

Sex steroids, ultimately derived from cholesterol, are synthesized predominantly in gonadal tissue, the adrenal gland and placenta as a consequence of enzymatic conversions. After peripheral metabolism, androgenic activity is represented in a variety of steroid molecules that include testosterone (**Figure 7**). There is evidence in a range of tissues that the eventual cellular effects of testosterone may not be the result (or not only the result) of direct action of testosterone, but may also reflect the effects of sex steroid metabolites formed as a consequence of local enzyme activities.

The most important testosterone metabolites in bone are 5α-DHT (the result of 5α reduction of testosterone) and estradiol (formed by the aromatization of testosterone). Testosterone and DHT are the major and most potent androgens, with androstenedione (the major circulating androgen in women) and DHEA as immediate androgen precursors that exhibit weak androgen activity (39). In men, the most abundant circulating androgen metabolite is testosterone while concentrations of other weaker androgens like androstenedione and DHEA-sulfate are similar between males and females. Downstream metabolites of DHT and androstenedione are inactive at the AR, and include 5α-androstane-3α or 3β,17β-diol (3α/β-androstanediol) and 5α-androstanedione. Data suggests that aromatase cytochrome P450 (the product of the CYP19 gene), 17β-hydroxysteroid dehydrogenase (17ß-HSD), and 5α-reductase activities are all present in bone tissue, at

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least to some measurable extent in some compartments, but the biologic relevance of each remains somewhat controversial.

4.1 5α-reductase activity in osteoblasts

5α-reductase is an important activity with regard to androgen metabolism in general, since testosterone is converted to the more potent androgen metabolite DHT via 5α-reductase action (117). 5α-reductase activity was first described in crushed rat mandibular bone (118) with similar findings reported in crushed human spongiosa (119). Two different 5α-reductase genes encode type 1 and type 2 isozymes in many mammalian species (120); human osteoblastic cells express the type 1 isozyme (121). Essentially the same metabolic activities were reported in experiments with human epiphyseal cartilage and chondrocytes (122). In general, the K_m values for bone 5α-reductase activity are similar to those in other androgen responsive tissues (33, 119). However, the cellular populations in many of these studies were mixed and hence the specific cell type responsible for the activity is unknown. Interestingly, Turner *et al.* found that periosteal cells do not have detectable 5α-reductase activity (123), raising the possibilities that the enzyme may be functional in only selected skeletal compartments, and that testosterone may be the active androgen metabolite at this clinically important site.

From a clinical perspective, the general importance of this enzymatic pathway is uncertain, as patients with 5α-reductase type 2 deficiency have normal bone mineral density (124) and Bruch *et al.* found no significant correlation between enzyme activities and bone volume (117). In mutant null mice lacking 5α-reductase type 1 (mice express very little type 2 isozyme), the effect on the skeleton has not been analyzed due to midgestational fetal death as a consequence of estrogen excess (125). Analysis of the importance of 5α-

reductase activity has been approached with the use of finasteride (an inhibitor of 5α-reductase activity); treatment of male animals does not recapitulate the effects of castration (126), strongly suggesting that reduction of testosterone to DHT by 5α-reductase is not the major determinant in the effects of gonadal hormones on bone. Consistent with this finding, testosterone therapy in hypogonadal older men, either when administered alone or when combined with finasteride, increases bone mineral density, again suggesting that DHT is not essential for the beneficial effects of testosterone on bone (127). Thus, the available clinical data remains uncertain, and the impact of this enzyme, which isozyme may be involved, whether it is uniformly present in all cell types involved in bone modeling/remodeling, or whether local activity is important at all, remain unresolved issues.

4.2 Aromatization of testosterone in bone

Another important enzymatic arm of testosterone metabolism involves the biosynthesis of estrogens from androgen precursors, catalyzed by aromatase. Of note, this enzyme is well known to be both expressed and regulated in a very pronounced tissue-specific manner (128), and also demonstrates species differences, given the low levels in mice. Modest levels of aromatase activity have been reported in bone from mixed cell populations derived from both sexes (129-131) and from osteoblastic cell lines (33, 132, 133). Aromatase expression in intact bone has also been documented by *in situ* hybridization and immunohistochemical analysis (131). Aromatase mRNA is expressed predominantly in lining cells, chondrocytes and some adipocytes, however there is no detectable expression in osteoclasts, or in cortical bone in mice (66). At least in vertebral bone, the mesenchymal distal promoter I.4 is predominantly utilized (134). The enzyme kinetics in bone cells seem to be similar to those in other tissues, although the V_{max} may be increased by glucocorticoids (133). Whether the level of aromatase activity in bone is high

enough to produce physiologically relevant concentrations of steroids remains an open question; nevertheless in the male only 15% of circulating estrogen is produced in the testes, with the remaining 85% produced by peripheral metabolism that could include bone as one site of conversion (135).

Aromatase catalyzes the metabolism of adrenal and testicular C19 androgens (androstenedione and testosterone) to C18 estrogens (estrone and estradiol), thus producing the potent estrogen estradiol (E2) from testosterone, and the weaker estrogen estrone (E1) from its adrenal precursors androstenedione and DHEA (129). Typically in the circulation, E2 will make up to 40 percent of total estrogen, E1 will make up an additional 40 percent, with estriol (E3) comprising the remaining 20 percent of total estrogen (136). In addition to aromatase itself, osteoblasts contain enzymes that are able to inter-convert estradiol and estrone (17β-HSD), and to hydrolyze estrone sulfate, the most abundant estrogen in the circulation, to estrone (steroid sulfatase) (132, 137). Nawata *et al*. have reported that dexamethasone and 1α,25(OH)$_2$D$_3$ synergistically enhance aromatase activity and aromatase mRNA expression in human osteoblast-like cells (129). In addition, both leptin and 1α,25(OH)$_2$D$_3$ treatment increased aromatase activity in human mesenchymal stem cells during osteogenesis, but not during adipogenesis (138). Additional studies are needed to better define expression, given the potential importance of the enzyme, and its regulation by a variety of mechanisms (including androgens and estrogens) in other tissues (128, 139).

The clinical impact of aromatase activity and an indication of the importance of conversion of circulating androgen into estrogen is shown in reports of women and men with aromatase deficiencies, who present with a skeletal phenotype (140). Interestingly, natural mutation is remarkably rare with only seven males and six females reported to date. The presentation of men with aromatase deficiency is very similar to that of a man with estrogen

receptor-α (ERα) deficiency (141), namely an obvious delay in bone age, lack of epiphyseal closure and tall stature with high bone turnover and osteopenia (135), suggesting that aromatase (and likely estrogen action) has a substantial role to play during skeletal development in the male. In addition, estrogen therapy of males with aromatase deficiency has been associated with an increase in bone mass (135) particularly in the growing skeleton (142). Inhibition of aromatization pharmacologically with nonsteroidal inhibitors (such as vorozole or letrozole) results in modest decreases in bone mineral density and changes in skeletal modeling in young growing orchidectomized males (143), and less dramatically so in boys with constitutional delay of puberty treated for one year (144), suggesting short term treatment during growth has limited negative consequences in males. Inhibition of aromatization in older orchidectomized males resembles castration with similar increases in bone resorption and bone loss, suggesting that aromatase activity likely plays a role in skeletal maintenance in males (145). These studies herald the importance of aromatase activity (and estrogen) in the mediation of some androgen action in bone in both males and females. The finding of these enzymes in bone clearly raises the difficult issue of the origin of androgenic effects in the skeleton; do they arise solely from direct androgen effects (as is suggested by the actions of nonaromatizable androgens such as DHT) or also from the local or other site production of estrogenic intermediates? The results described above would seem to indicate that both steroids appear to be important to both male and female skeletal health.

4.3 17β-hydroxysteroid dehydrogenase activity in osteoblasts

   The 17β-HSDs (most of which are dehyrogenase-reductases, except type 5 that is an aldo-keto reductase) have been shown to either catalyze the last step of sex steroid

synthesis or the first step of their degradation (to produce weak or potent sex steroids via oxidation or reduction, respectively), and can thus also play a critical role in peripheral steroid metabolism. The oxidative pathway forms 17-ketosteroids while the reductive pathway forms 17β- hydroxysteroids. The enzyme reversibly catalyzes the formation of androstenediol (an estrogen) from DHEA, in addition to the biosynthesis of estradiol from estrone, the synthesis of testosterone from androstenedione, and the production of DHT from 5α-androstanedione all via the reductive activity of 17β-HSD. Of the 13 enzyme isotypes of 17β-HSD activity (136), types 1-4 have been demonstrated in human osteoblastic cells (146).

The administration of testosterone can stimulate bone formation and inhibit bone resorption, likely through multiple mechanisms that involve both androgen and estrogen receptor-mediated processes. However, there is substantial evidence that some, if in fact not most, of the biologic actions of androgens in the skeleton are mediated by AR. Both *in vivo* and *in vitro* systems reveal the effects of the nonaromatizable androgen DHT to be essentially the same as those of testosterone (*vida infra*). In addition, blockade of the AR with the receptor antagonist flutamide results in osteopenia as a result of reduced bone formation (5). In addition, complete androgen insensitivity results in a significant decrease in bone mineral density in spine and hip sites (124) even in the setting of strong compliance with estrogen treatment (147). These reports clearly indicate that androgens, independent of estrogenic metabolites, have primary effects on osteoblast function. However, the clinical reports of subjects with aromatase deficiency also highlight the relevance of metabolism of androgen to bio-potent estrogens at least in the circulation, to influence bone development and/or maintenance. It thus seems likely that further elucidation of the regulation of steroid metabolism, and the potential mechanisms by which androgenic and estrogenic effects are coordinated, will have physiological, pathophysiological, and therapeutic implications.

4.4 Drugs with androgenic activity

In addition to the endogenous steroid metabolites highlighted in **Figure 7**, there are also a variety of drugs with androgenic activity. These include anabolic steroids, such as nonaromatizable oxandrolone, that bind and activate AR (albeit with lower affinity than testosterone (148)), and a class of drugs under extensive development referred to as SARMs, that demonstrate tissue-specific agonist or antagonist activities with respect to AR transactivation (149). These orally active nonsteroidal nonaromatizable SARMS are being developed to target androgen action in bone, muscle, fat and to influence libido but to not exacerbate prostate growth, hirsutism and acne. Several have recently been identified with beneficial effects on bone mass (150-152), and provide a new alternative to androgen replacement therapy.

5. Gender-specificity in the actions of sex steroids

Although controversial, there may be gender specific responses in osteoblastic cells to sex steroids. In most mammals, there is a marked gender difference in morphology that results in a sexually dimorphic skeleton. The mechanisms responsible for these differences are necessarily complex, and presumably involve both androgenic and estrogenic actions on the skeleton. It is becoming increasingly clear that estrogens are particularly important for the regulation of epiphyseal function and act to reduce the rate of longitudinal growth via influences on chondrocyte proliferation and function, as well as on the timing of epiphyseal closure (153). Androgens, on the other hand, appear to have many opposite effects to estrogen on the skeleton. For example, androgens tend to promote long bone growth, chondrocyte maturation, and metaphyseal ossification, opposite to effects of estrogen.

Another notable example is the effect of AR activation in cortical bone in males, which can stimulate bone formation at the periosteal surface but inhibit formation at the endosteum (66). Thus, the most dramatic effect of androgens is on bone size, in particular cortical thickness (154). This difference of course has important biomechanical implications, with thicker bones being stronger bones (155). Furthermore, the response of the adult skeleton (to the same intervention) results in distinct responses in males and females. For example, in a model of disuse osteopenia, antiorthostatic suspension results in significant reduction in bone formation rate at the endosteal perimeter in males. In females however, a decrease in bone formation rate occurred along the periosteal perimeter (156). Gender-specific responses *in vivo* and *in vitro* (for example, see 86), and the mechanism(s) that underlie such responses in bone cells, may thus have significant implications in treatment options for metabolic bone disease.

6. Conclusion

Thus, the effects of androgens on bone health are both complex and pervasive. Androgens influence skeletal modeling and remolding by multiple mechanisms through effects on osteoblasts, osteoclasts and even perhaps an influence on the differentiation of pluripotent stem cells toward distinct lineages. The specific effects of androgen on bone cells are mediated directly through an AR-signaling pathway, but there are also indirect contributions to overall skeletal health through aromatization and ER signaling. The effects of androgens are particularly dramatic during growth in boys particularly at the periosteum, but almost certainly play an important role during this period in girls as well. Throughout the rest of life, androgens affect skeletal function and maintenance in both sexes. Nevertheless, given this importance, relatively little has been done to unravel the mechanisms by which androgens influence the physiology and pathophysiology of bone, and there is still much to

be learned about the roles of androgens at all levels. The interaction of androgens and estrogens, and how their respective actions can be utilized for specific diagnostic and therapeutic benefit, are important but unanswered issues. With an increase in the understanding of the nature of androgen effects will come greater opportunities to use their positive actions in the prevention and treatment of a wide variety of bone disorders.

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Figure Legends.

Figure 1. Model of AR regulation of gene expression. Binding of androgen promotes high-affinity dimerization, followed by DNA binding at the androgen response element (ARE) in an androgen-responsive gene promoter. Coactivators may remodel chromatin through histone acetylase activity to open chromatin structure (157), or act as a bridge to attract transcription factors (TFs) that target binding of TATA-binding protein to the TATAA sequence (13). Phosphorylation of receptor may result from activation of SRC by growth factors (22). Smad3 can act as either a coactivator or corepressor (158, 159), while cyclin D1 is a corepressor of AR transactivation (21). AR can also directly contact TFIIH and TFIIF (160) in the general transcription machinery. Such interactions between the AR and the general transcription machinery, leading to stable assembly, results in recruitment of RNA polymerase II and subsequent increased gene transcription. Downregulation of gene expression can also be AR mediated.

Figure 2. The localization of AR in normal tibial growth plate and adult osteophytic human bone. a) Morphologically, sections of the growth plate consist of areas of endochondral ossification with undifferentiated (*small arrow head*), proliferating (*large arrow heads*), mature (*small arrow*) and hypertrophic (*large arrow*) chondrocytes. Bar = 80 µm. An inset of an area of the primary spongiosa is shown in b. b) Numerous osteoblasts (*small arrow heads*) and multinucleated osteoclasts (*large arrow heads*) on the bone surface. Mononuclear cells within the bone marrow are also present (*arrows*). Bar = 60 µm. c) In the growth plate, AR is predominantly expressed by hypertrophic chondrocytes (*large arrow heads*). Minimal expression is observed in the mature chondrocytes (*small arrow heads*). The receptors are rarely observed in the proliferating chondrocytes (*arrow*). d) In the primary spongiosa, the

AR is predominantly and highly expressed by osteoblasts at modeling sites (*arrow heads*).
Bar = 20 µm. e) In the osteophytes, AR is also observed at sites of endochondral
ossification in undifferentiated (*small arrow heads*), proliferating (*large arrow heads*), mature
(*small arrows*), and hypertrophic-like (*large arrow*) chondrocytes. Bar = 80 µm. f) A higher
magnification of e) showing proliferating, mature, and hypertrophic-like chondrocytes (*large
arrows, small arrows,* and *very large arrows* respectively) Bar = 40 µm. g) At sites of bone
remodeling, the receptors are highly expressed in the osteoblasts (*small arrow heads*) and
also in mononuclear cells in the bone marrow (*large arrow heads*). Bar = 40 µm. h) AR is
not detected in osteoclasts (*small arrow heads*) Bar = 40 µm. B, Bone: C, Cartilage; BM,
Bone marrow. Adapted from Abu *et al* (10) and used with permission.

Figure 3. Dichotomous regulation of AR mRNA levels in osteoblast-like and prostatic
carcinoma cell lines after exposure to androgen. A, Time course of changes in AR mRNA
abundance after DHT exposure in human SaOS-2 osteoblastic cells and human LNCaP
prostatic carcinoma cells. To determine the effect of androgen exposure on hAR mRNA
abundance, confluent cultures of either osteoblast-like cells (SaOS-2) or prostatic carcinoma
cells (LNCaP) were treated with 10^{-8} M DHT for 0, 24, 48, or 72 h. Total RNA was then
isolated and subjected to RNase protection analysis with 50 µg total cellular RNA from
SaOS-2 osteoblastic cells and 10 µg total RNA from LNCaP cultures. B, Densitometric
analysis of AR mRNA steady state levels. The AR mRNA to β-actin ratio is expressed as the
mean $\pm$ SE compared to the control value from three to five independent assessments.
Adapted from Wiren *et al* (73) and used with permission.

Figure 4. Expression analyses of ERα, ERß and AR during *in vitro* differentiation in normal rat osteoblastic (rOB) cultures. (A) Normal rOB cells were cultured for the indicated number of days during proliferation, matrix maturation, mineralization and postmineralization stages. Total RNA was isolated and subjected to relative RT-PCR analysis using primers specific for rat ERα, ERß and AR or rat GAPDH. Reverse transcription was conducted with PCR carried out for 40 cycles for the steroid receptors, with parallel reactions performed using GAPDH primers for 25 cycles (all in the linear range). Bands for rat ERα at the predicted 240 bp, rat ERß at 262 bp, rat AR at 276 bp and GAPDH at 609 bp are shown. (B) Analyses of ERα, ERß and AR mRNA relative abundance. Semi-quantitative analysis of mRNA steady-state expression by relative RT-PCR was performed after scanning the negative image of the photographed gels. Data are expressed in arbitrary units as the ratio of receptor abundance to GAPDH expression, then normalized to expression values at day 4 in pre-confluent cultures. Data represent mean ± SEM. Adapted from Wiren *et al* (78) and used with permission.

Figure 5. Complex effect of androgen on DNA accumulation in osteoblastic cultures. Kinetics of DHT response in proliferating colAR-MC3T3 cultures measured with colorimetric (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay. Cultures of stably transfected colAR-MC3T3 continuously with 10^{-8} M DHT for 2 days led to increased MTT accumulation, but longer treatment for 3 or 5 days resulted in inhibition. Data are mean ± SEM of six to eight dishes with six wells/dish. *$p < 0.05$; **$p < 0.01$ (vs control). Adapted from Wiren *et al* (87) and used with permission.

Figure 6. Characterization of osteoblast apoptosis: results of androgen and estrogen treatment during proliferation (day 5) and during differentiation into mature osteoblast/osteocytes cultures (day 29). Apoptosis was assessed at day 5 or day 29 after continuous DHT and E_2 treatment (both at 10^{-8} M). Apoptosis was induced by etoposide treatment in proliferating cultures and by serum starvation for 48 h in confluent cultures before isolation, replaced with 0.1% BSA. (A) Analysis of apoptosis after evaluating DNA fragmentation by cytoplasmic nucleosome enrichment at day 5. The data are expressed as mean ± SEM (n=6) from two independent experiments. **$p < 0.01$, ***$p < 0.001$ (vs. control). (B) Analysis of apoptosis by cytoplasmic nucleosome enrichment analysis at day 29. The data are expressed as mean ± SEM (n=6) from two independent experiments. **$p < 0.01$ vs. control.   Adapted from Wiren *et al* (92) and used with permission.

Figure 7. Principle conversions and major enzyme activities involved in androgen synthesis and metabolism. Steroid hormone synthesis involves metabolism of cholesterol, with dehydrogenation of pregnenolone producing progesterone that can serve as a precursor for the other gonadal steroid hormones. DHEA, dehydroepiandrosterone; CYP11A, cytochrome P450 cholesterol side chain cleavage enzyme; CYP17, cytochrome P450 17α hydroxylase/17,20 lyase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; CYP19, aromatase cytochrome P450.

Figure 1. Model of steroid receptor regulation of gene expression.